

**UNIVERSITA' DEGLI STUDI DI NAPOLI
"FEDERICO II"**

**DOTTORATO DI RICERCA IN GENETICA
E MICROBIOLOGIA CELLULARE E
MOLECOLARE**

TESI

**"LE TIROSINO FOSFATASI SHP-2 E SHP-1
FORMANO DUE DISTINTI COMPLESSI
CON LE FORME ATTIVATE DI RET E NE
MEDIANO LA TRASMISSIONE DEL
SEGNALE INTRACELLULARE"**

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**UNIVERSITA' DEGLI STUDI DI NAPOLI
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PATOLOGIA CELLULARE E
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ABBREVIAZIONI

CLD. *Cadherine like domainn*

CREB. *Cyclic AMP response-element binding protein*

DOK 4/5. *Downstream of tyrosine kinase protein*

EGF. *Epidermal growth factos*

Erk. *Extracellular regulated kinase*

FGF. *Fibroblast growth factor*

FMTC. *Familial Medullary Thyroid Carcinomas*

FRS2. *Fibroblast growth factor receptor substrate 2*

Gab1. *Grb-associated binding protein 1*

Gab2. *Grb-associated binding protein 2*

GDNF. *Glial cell line-derived neutrophic factor*

GFR α . *GDNF family receptor alfa*

Grb2. *Growth factor receptor bound protein 2*

IGF-1. *Insulin-like growth factor-1*

JNK. *c-jun NH2-terminal kinase*

MAPK. *Mitogen-activated protein kinase*

MEN 2. *Multiple Endocrine Neoplasia type 2*

NF-kB. *Nuclear factor kappa B*

NGF. *Nerve growth factor*

PDGF. *Platelet-derived growth factor*

PI3K. *Phosphatidylinositol 3-kinase*

PTP. *Phospho tyrosine phosphatase*

RET. *REarranged during Trasfection*

SHC. *SH2-containing protein*

Shp-1. *Src homology 2-domain containing phosphatase 1*

Shp-2. *Src homology 2-domain containing phosphatase 2*

Spry. Sprouty

SOMMARIO

La Neoplasia Endocrina Multipla (MEN) di tipo 2A di tipo 2B e il carcinoma midollare familiare della tiroide (FMTC) sono sindromi tumorali familiari clinicamente distinte, tutte caratterizzate dall'insorgenza del carcinoma midollare della tiroide e, nel caso della MEN 2A e MEN 2B, del feocromocitoma. Da oltre 10 anni è noto che le MEN 2 e il FMTC sono sindromi tumorali causate da un numero relativamente limitato di mutazioni geniche puntiformi dell'oncogene *ret*, il quale codifica per un recettore di membrana con attività tirosino chinasi. Queste mutazioni conferiscono al recettore uno stato di attivazione costitutiva ligando-indipendente con conseguente proliferazione neoplastica degli organi bersaglio. In condizioni fisiologiche, l'attività tirosino chinasi del recettore RET è stimolata da ciascuno dei quattro ligandi appartenenti alla famiglia del *glial cell line-derived neurotrophic factor* (GDNF) che ne inducono una rapida attivazione.

La trasduzione del segnale iniziata da RET è necessaria per lo sviluppo e la sopravvivenza di popolazioni neuronali del sistema nervoso centrale e periferico, per la morfogenesi del rene, e per la differenziazione degli spermatogoni.

Il corretto funzionamento dei meccanismi di trasduzione del segnale si basa sul bilancio fra l'attività biologica degli enzimi che catalizzano reazioni di

fosforilazione di proteine bersaglio (le chinasi) e quelle degli enzimi che ne catalizzano la reazione inversa, defosforilazione (le fosfatasi).

Negli ultimi anni, è stata ampiamente descritta l'attivazione di chinasi, incluso l'attivazione di Raf, Erk, c-Src, PI3-kinasi, AKT e JNK, coinvolte nelle vie di trasduzione del segnale dipendente da RET. Poco invece ancora si conosce sul coinvolgimento delle fosfatasi in queste stesse cascate enzimatiche.

Allo scopo di determinare il ruolo di specifiche proteine fosfatasi nelle vie di trasduzione del segnale mediate da RET, in questo lavoro abbiamo studiato la possibile implicazione di due tirosino fosfatasi, Shp-1 e Shp-2, come mediatori nei segnali intracellulari attivati dal recettore sia in forma *wild type* (in condizioni fisiologiche) che in forma mutata (nelle patologie MEN 2).

L'interesse nello studio di Shp-1 e Shp-2 scaturisce dalla evidenza che, sebbene le due fosfatasi siano molto simili nella struttura, in molti sistemi biologici esse svolgono funzioni opposte. Shp-1 funziona generalmente come effettore negativo in molte vie di trasduzione del segnale, sia iniziate da citochine che da recettori ad attività tirosino chinasi. Viceversa, la funzione di Shp-2 è necessaria alla trasmissione di segnali extracellulari all'interno di una cellula.

Per i nostri studi abbiamo utilizzato come sistema modello due linee cellulari originate per trasfezione da cellule PC12 (derivate da

feocromocitoma sperimentale di ratto) che esprimono sia la fosfatasi Shp-1 che la fosfatasi Shp-2. Le PC12 sono state trasfettate stabilmente con un vettore di espressione per il recettore umano mutato, Ret^{C634Y} o Ret^{M918T} (rispettivamente indicate come cellule PC12/Ret^{C634Y} e PC12/Ret^{M918T}). La mutazione della cisteina 634 è associata alla MEN 2A mentre la mutazione della metionina 918 in treonina è la più frequente nella MEN 2B.

Abbiamo dimostrato che Shp-1 e Shp-2 interagiscono con le forme attivate costitutivamente di RET, attraverso la formazione di un complesso multiproteico di membrana. Inoltre, mediante centrifugazione su gradiente di saccarosio, abbiamo dimostrato che Shp-1 interagisce con le forme attivate di RET preferenzialmente in specifici microdomini di membrana che prendono il nome di “lipid rafts”, mentre il complesso di Shp-2 con RET è localizzato solo ed esclusivamente al di fuori dei lipid rafts. L’interazione di entrambe le fosfatasi con RET attivato non è diretta e RET non è substrato né di Shp-1 né di Shp-2. I nostri risultati dimostrano che Shp-2 è fondamentale per la differenziazione e la proliferazione di cellule PC12 mediate dal recettore RET.

L’evidenza che, nelle nostre condizioni sperimentali, Shp-1 e Shp-2 legano RET in microdomini di membrana distinti potrebbe spiegare l’osservazione generale che le due fosfatasi svolgono funzioni biologiche opposte. A conferma di questa ipotesi è noto che per molte proteine, la differente distribuzione all’interno e all’esterno dei “lipid rafts”, regola il loro

coinvolgimento in molte vie di trasduzione del segnale che includono l'apoptosi, l'adesione e la migrazione cellulare, la trasmissione sinaptica, l'organizzazione del citoscheletro e il *sorting* in esocitosi e endocitosi.

INTRODUZIONE

IL RECETTORE TIROSINO CHINASI RET

La crescita, la differenziazione e le funzioni di cellule neuroendocrine sono controllate da una varietà di fattori che includono fattori di crescita, fattori neurotrofici e citochine. Questi stimoli extracellulari esercitano funzioni biologiche attraverso specifici recettori espressi sulle cellule bersaglio. Il recettore RET è stato identificato per la prima volta nel 1985 come un oncogene attivato mediante riarrangiamenti del DNA (Takahashi *et al.* 1985; 1987). Il protooncogene *ret* codifica un recettore ad attività tirosino chinasi espresso, durante lo sviluppo embrionale, nelle cellule della cresta neurale e nei precursori delle cellule urogenitali (Avantaggiato *et al.* 1994). La sua attività è essenziale per lo sviluppo del sistema nervoso enterico e renale durante l'embriogenesi (Jijiwa *et al.* 2004). Inoltre, alleli con mutazioni *gain of function* di *ret*, sono stati identificati come i responsabili di sindromi tumorali familiari come il carcinoma papillare della tiroide e la neoplasia endocrina multipla di tipo 2, mentre alleli con mutazioni *loss of function* sono responsabili della malattia di Hirschsprung (Grieco *et al.* 1990; Mulligan *et al.* 1993; Romeo *et al.* 1994).

La struttura del recettore RET

Il gene *ret* è localizzato sul cromosoma 10 (10q11.2) e comprende 21 esoni (Takahashi *et al.* 1988; 1989; Iwamoto *et al.* 1993). Come conseguenza di un meccanismo di splicing alternativo nella regione 3' del trascritto, si formano due isoforme del gene *ret*, l'isoforma corta (RET9) e la lunga (RET51), le quali differiscono nella sequenza amminoacidica all'estremità C-terminale (Tahira *et al.* 1990; Myers *et al.* 1995). Sono stati generati topi che esprimono solo RET9 o solo RET51 ed è stato dimostrato che RET9 è cruciale per la morfogenesi del rene e per lo sviluppo del sistema nervoso enterico; i topi che esprimono solo l'isoforma RET9 apparentemente crescono normalmente, mentre i topi che esprimono solo l'isoforma RET51 soffrono di ipoplasia del rene e mancano dei gangli enterici del colon (De Graaff *et al.* 2001). D'altra parte, da studi *in vitro* è stato dimostrato che solo l'isoforma RET51 può promuovere la sopravvivenza e la tubulogenesi delle cellule del colon. In complesso, i risultati riportati suggeriscono che i segnali del recettore nell'isoforma RET51 partecipano ad eventi responsabili della fase tardiva dell'organogenesi del rene, mentre quelli che partono dall'isoforma RET9 sono indispensabili nelle fasi iniziali dello sviluppo del rene (Ichihara *et al.* 2004; Lee *et al.* 2002).

Come tutte le proteine transmembrana, i recettori tirosino chinasi sono costituiti da una regione extracellulare, da una regione che attraversa il "bilayer" lipidico e da una regione intracellulare. La regione extracellulare,

che coincide con l'estremità N-terminale della proteina, è costituita da 636 aminoacidi e contiene il sito di interazione con il ligando. A differenza di altri recettori tirosino-chinasici, che in questa regione possiedono domini simili a quelli delle immunoglobuline e della fibronectina, la proteina RET possiede una ripetizione in tandem di quattro domini simili alle caderine (*cadherine like domains*, CLD1-4) (Anders *et al.* 2001), proteine che mediano l'adesione cellula-cellula. Le caderine legano ioni Ca^{2+} e vengono da questi irrigidite, stabilizzate e protette dalla degradazione proteolitica. E' stato dimostrato che i quattro domini "cadherine-like" di RET sono necessari per la corretta maturazione post-traduzionale del recettore, infatti, le mutazioni nel gene *ret* che colpiscono i domini CLDs del recettore sono causa di alterazioni del folding, della glicosilazione e del trasporto della proteina in membrana. Come le caderine, RET è in grado di legare ioni Ca^{2+} , che inducono una modificazione conformazionale della regione extracellulare, e stabilizzano la proteina rendendola più resistente all'azione proteolitica (Anders *et al.* 2001). Sebbene i domini CLDs formino una grossa porzione della regione extracellulare di RET, l'estremità N-terminale della proteina contiene altri due domini importanti: la sequenza aminoacidica responsabile del trasporto della proteina sulla membrana "leader peptide" e una regione ricca in residui di cisteina (CRD) localizzata nella porzione prossimale alla membrana plasmatica. La regione ricca in cisteine contiene 28 residui di questo aminoacido, alcuni dei quali sono

impegnati nella formazione di ponti disolfuro, e coinvolti nella formazione e nel mantenimento della struttura terziaria. Mutazioni di alcune cisteine presenti in questa regione, ad esempio la sostituzione del residuo di cisteina 634, sono ereditate nelle sindrome MEN 2A e FMTC e causano l'attivazione costitutiva di RET.

La regione intracellulare di RET comprende gli aminoacidi che vanno dal 726 all'estremità C-terminale ed è costituita da due domini tirosino-chinasici (Tk-a e Tk-b) mediante i quali RET, in seguito alla dimerizzazione, è in grado di autofosforilarsi a livello di specifici residui di tirosina. L'attivazione del dominio catalitico ed il riconoscimento delle fosfo-tirosine da parte di specifiche proteine è il meccanismo che innesca la cascata intracellulare di trasduzione del segnale mediato da recettore RET (figura 1).

Il meccanismo di attivazione di RET

I ligandi specifici di RET appartengono alla famiglia del GDNF (*glial cell line-derived neurotrophic factor*) e stimolano la maturazione e la sopravvivenza di cellule neuronali, la morfogenesi del rene e la divisione degli spermatozoi (Baloh *et al.* 2000). La famiglia GDNF comprende il GDNF stesso, la Neurturina, l'Artemina, e la Persefina. Tutti i ligandi sono secreti come omodimeri e utilizzano RET come recettore per trasmettere segnali all'interno della cellula. Una delle caratteristiche che distingue RET

dagli altri recettori tirosino-chinasici è l'impossibilità di interagire con i propri ligandi senza l'ausilio di co-recettori, proteine appartenenti alla famiglia del $\text{GFR}\alpha$ (*growth factor receptor-alfa*). I co-recettori formano con RET e con il fattore di crescita un complesso che è in grado di attivare il recettore e di trasdurre il segnale all'interno della cellula. La famiglia dei $\text{GFR}\alpha$ è formata da quattro differenti recettori extracellulari ancorati alla membrana da una molecola di glicosil-fosfatidilinositolo (GPI). Ciascuna molecola di co-recettore lega in maniera selettiva, ma non totalmente esclusiva, uno dei fattori di crescita appartenenti alla famiglia del GDNF (figura 2). Il GDNF lega con elevata affinità il co-recettore $\text{GFR}\alpha$ -1 e con affinità più bassa il $\text{GFR}\alpha$ -2; la Neurturina interagisce con il $\text{GFR}\alpha$ -2 e con minore affinità con il $\text{GFR}\alpha$ -1; l'Artemina è in grado di legare il $\text{GFR}\alpha$ -3 ma anche il $\text{GFR}\alpha$ -1; la Persefina lega esclusivamente il $\text{GFR}\alpha$ -4 (Airaksinen *et al.* 2002; Manie *et al.* 2001). Per effetto di un meccanismo di *splicing* alternativo, l'mRNA del $\text{GFR}\alpha$ -1 produce due isoforme: $\text{GFR}\alpha$ -1a e $\text{GFR}\alpha$ -1b. Abbiamo dimostrato che l'isoforma $\text{GFR}\alpha$ -1a è espressa soprattutto in tessuti neuronali, è poco rappresentata prima della nascita mentre risulta essere l'isoforma prioritaria dopo la nascita. Studi *in vitro* hanno indicato che l'isoforma $\text{GFR}\alpha$ -1b lega il GDNF con più efficienza rispetto all'isoforma $\text{GFR}\alpha$ -1a (Chalet-Berguerand *et al.* 2004). La composizione stechiometrica del complesso multiproteico RET/ $\text{GFR}\alpha$ -

1/GDNF è 2:2:2 (figura 3). Nel nostro laboratorio, mediante esperimenti di *crosslinking* tra RET, GFR α -1 e GDNF in forma solubile, seguiti da SDS-PAGE dei complessi covalenti, digestione triptica e analisi per MALDI MS *fingerprinting*, sono stati identificati i domini coinvolti nel legame tra le tre proteine (Amoresano *et al.* 2004). In accordo con quanto è riportato in letteratura, non sono stati evidenziati complessi che includono RET e il GDNF in assenza di GFR α -1. Inoltre abbiamo dimostrato che i domini CLD4 e CRD di RET sono necessari per la formazione di un complesso trivalente biologicamente attivo.

Dopo il legame con un dimero di ligando, il recettore RET subisce una dimerizzazione ed una trans-fosforilazione a livello di specifici residui di tirosina localizzati nella regione intracellulare, tappa indispensabile per l'attivazione della sua intrinseca attività tirosino chinasi. La isoforma RET9 contiene 16 residui di tirosina che comprendono 10 residui nel dominio catalitico, 2 residui nella regione intracellulare immediatamente sotto la membrana plasmatica, 1 residuo nell'inserto del dominio chinasi e 3 residui nella coda C-terminale, mentre l'isoforma RET51 contiene due residui di tirosina in più nella coda C-terminale. Tra questi residui, le tirosine in posizione 905, 1015, 1062, e 1096 rappresentano siti di legame per diverse proteine intracellulari alcune delle quali sono la proteina Grb7/10 (*growth factor receptor bound protein 7/10*), la fosfolipasi C γ (PLC γ) e le proteine adattatrici SHC (*SH2-containing protein*) e Grb2 (Asai

et al. 1996; Borrello *et al.* 1996; Arighi *et al.* 1997) (figura 1). Le tre tirosine in posizione 905, 1015, e 1062 sono conservate in tutte e due le isoforme di RET mentre la tirosina 1096 è presente solo nell'isoforma RET51. Inoltre, nelle isoforme RET9 e RET51, è diverso il *consensus motif* della tirosina in posizione 1062.

LE MUTAZIONI DEL GENE *RET* E LE PATOLOGIE ASSOCIATE

Il gene *ret* è stato isolato come forma oncogenica chimerica durante un dosaggio di trasformazione di cellule NIH 3T3. Per tale motivo è stato chiamato RET (“REarranged during Trasfection”) (Takahashi *et al.* 1985)

Il gene *ret* è caratterizzato da una straordinaria eterogeneità allelica: a seconda della localizzazione della mutazione all'interno del gene, e quindi del dominio proteico colpito, gli alleli mutati possono codificare proteine con fenotipo “perdita di funzione” (mutanti *loss of function*) o proteine attivate costitutivamente (mutanti *gain of function*).

Alleli di *ret* con mutazioni “loss of function”

Nella sequenza codificante di RET sono state identificate diverse mutazioni puntiformi e frameshift. La maggior parte di queste mutazioni inattiva il recettore, ed è responsabile dello sviluppo di una patologia molto severa, la malattia di Hirschsprung (HSCR) (Edery *et al.* 1994). La malattia HSCR è

un disordine congenito caratterizzato dalla assenza dei plessi nervosi intramurali del tratto gastrointestinale, provocata dall'arresto prematuro della migrazione di neuroni enterici dalla cresta neurale al segmento gastroenterico. La sintomatologia della HSCR è spesso associata con neurocrestopatie come il morbo di Waardenburg, le sindromi pigmentarie, la MEN2 e con difetti del tubo neurale. I geni specifici che possono essere responsabili di questa patologia sono stati identificati grazie ad analisi di "positional cloning", studi di linkage e analisi di mutazioni nei geni candidati. Oltre al gene *ret* sono stati ancora identificati come responsabili il gene *ednrb* che codifica una "G-coupled protein", il gene *edn3* che codifica il suo ligando e il gene *sox10* che codifica un fattore di trascrizione specifico dello sviluppo. Ciò dimostra che tutti questi geni codificano proteine che svolgono un ruolo fondamentale nello sviluppo del sistema nervoso (Manie *et al.* 2001; Takahashi *et al.* 1999). Le differenti patologie che insorgono in seguito a mutazioni nei geni elencati sopra suggeriscono che esista una fitta rete di interazioni fra proteine che, se mutata in una delle sue componenti, provoca un cattivo funzionamento dell'intero sistema di cellule appartenenti alla linea neuronale.

Alleli di *ret* con mutazioni "gain of function"

Le mutazioni nel gene *ret* che producono alleli *gain of function* possono essere ereditate attraverso cellule gametiche (mutazioni germinali) o avvenire in cellule somatiche.

Gli alleli mutati di *ret* del tipo “gain of function”, nonostante codifichino per un oncogene dominante con attività tirosino-chinasica, non sono letali se sono trasmessi per via germinale. Queste mutazioni sono la causa delle sindromi tumorali ereditarie MEN 2A e MEN 2B. La sindrome tumorale MEN 2A è provocata dalla sostituzione di un residuo di cisteina (in posizione 609, 611, 618, 620, oppure 634), localizzato nel dominio extracellulare della proteina, con un diverso residuo amminoacidico. Per effetto di questa mutazione, un residuo di cisteina normalmente impegnato in un legame disolfuro intramolecolare resta libero di formare un legame intermolecolare con un residuo di cisteina presente su un altro monomero di RET mutato.

La formazione di ponti disolfuro intermolecolari favorisce la dimerizzazione, e quindi l'attivazione costitutiva, del recettore. La sindrome MEN 2A provoca sviluppo di carcinomi midollari della tiroide, feocromocitomi e iperplasia delle paratiroidi (Hansford *et al.* 2000).

La sindrome MEN 2B (nel 98% dei casi) è causata dalla sostituzione, nel dominio tirosino-chinasico del recettore, di un residuo di metionina in posizione 918 con il residuo di treonina. Per effetto di questa mutazione, è codificata una proteina il cui dominio catalitico è attivato costitutivamente

anche in assenza di dimerizzazione. La proteina mutata induce la fosforilazione di substrati in assenza di dimerizzazione e una modificazione dell'affinità per il substrato. La sindrome tumorale MEN 2B comporta lo sviluppo di carcinomi midollari della tiroide, di feocromocitomi, ganglioneuromi enterici e la comparsa di anomalie scheletriche (Liu *et al.* 1996) .

La sindrome tumorale FMTC (*Familial Medullary Thyroid Carcinomas*) può essere associata sia a mutazioni nel dominio extracellulare che a mutazioni nel dominio intracellulare del recettore. Alcune delle mutazioni responsabili di questa sindrome coincidono con quelle responsabili di sindrome MEN 2A, ma il meccanismo con il quale la proteina mutata possa dare origine a patologie differenti non è conosciuto. La FMTC, a differenza delle altre sindromi tumorali prodotte da mutazioni nel gene *ret*, è meno severa e presenta esclusivamente sviluppo di carcinoma midollare della tiroide (figura 4).

Le mutazioni somatiche del gene *ret* comprendono riarrangiamenti genomici, che comportano fusione del 3' del gene con tratti di diversi geni eterologhi. La fusione genera oncogeni chimerici denominati RET\PTC (Jhian *et al.* 2000). La formazione di oncogeni RET/PTC è stata osservata in carcinomi papillari della tiroide, nei quali le proteine chimeriche svolgerebbero un ruolo chiave per lo sviluppo della patologia tumorale. La sostituzione del promotore fisiologico di *ret*, che non è normalmente

espresso in cellule epiteliali della tiroide, con quello del gene col quale è avvenuta la fusione determina l'espressione ectopica e non regolata del recettore.

La cascata intracellulare del segnale innescata dalla stimolazione di RET

L'autofosforilazione in tirosina di RET innesca il reclutamento e l'attivazione di proteine bersaglio intracellulari contenenti domini SH2 (*Src Homology-2-domain*) e domini PTB (*Phospho-Tyrosine Binding domain*). Questo riconoscimento è determinato da una interazione diretta tra le tirosine fosforilate del recettore e i domini SH2 e i domini PTB che sono presenti sia in proteine adattatrici che in proteine dotate di attività enzimatica (figura 5).

Il residuo di tirosina fosforilato che svolge il ruolo principale nel legame con le proteine adattatrici è quello in posizione 1062, che viene riconosciuto e legato dalle proteine SHC, FRS2 (*fibroblast growth factor receptor substrate 2*), DOK4/5 (*downstream of tyrosine kinases*), IRS1/2 (*insulin receptor substrate 1/2*) ed Enigma. Come molti recettori ad attività tirosino chinasi, RET può attivare diverse vie di traduzione del segnale che includono l'attivazione di Erk (*extracellular regulated kinase*) via Ras, AKT via PI3K (*Phosphatidylinositol 3-kinase*) e la via di JNK (*c-jun NH2-terminal kinase*) (Califano *et al.* 1996; Rizzo *et al.* 1996; Chiariello *et al.* 1998; Melillo *et al.* 1999; Hayashi *et al.* 2000; Califano *et al.* 2000;). L'aspetto interessante di questi dati è che per l'attivazione delle vie Ras/Erk, PI3K/AKT, p38MAP chinasi e JNK è necessaria la fosforilazione

del residuo di tirosina 1062 di RET. Infatti, in motoneuroni MN1, in seguito alla stimolazione con il GDNF, la proteina adattatrice SHC si lega alla fosfo-tirosina 1062 di RET e questo legame induce la formazione di un complesso multiproteico contenente RET, le proteine adattatrici SHC, Gab2 (*Grb2-associated binding protein 2*) e Grb2, la subunità p85 di PI3K e la fosfatasi Shp-2 (*Src homology two-domain containing phosphatase 2*). Questa interazione proteica è richiesta per l'attivazione delle vie PI3K/AKT (Hayashi *et al.* 2000; Besset *et al.* 2000). È stato dimostrato che l'attivazione delle vie Ras/Erk e PI3K/Akt attraverso la fosforilazione della tirosina 1062 è importante anche per l'attivazione di fattori trascrizionali come CREB (*cyclic AMP response-element binding protein*) ed NF-κB (*nuclear factor kappa B*) (Hayashi *et al.* 2000) e per la sopravvivenza di cellule PC12 (feocromocitoma di ratto) (De Vita *et al.* 2000). Nel nostro laboratorio è stato dimostrato che l'attivazione di Ras indotta da RET è essenziale per la differenziazione delle cellule PC12 (Califano *et al.* 2000).

L'interazione tra i “lipid rafts” e il recettore RET

La membrana plasmatica presenta una composizione lipidica che è molto differente dalla composizione di altre membrane della cellula. Tutte le membrane cellulari contengono i fosfolipidi che sono importanti per la formazione del *bilayer* di membrana, tuttavia la membrana plasmatica di cellule eucariotiche è costituita da altre due classi lipidiche che strutturano la membrana plasmatica in microdomini: gli steroli e gli sfingolipidi.

I primi domini lipidici di membrana ad essere identificati sono state le caveole, che tutt'ora rappresentano gli unici domini identificabili dal punto di vista morfologico. Sono strutturate come invaginazioni della membrana

plasmatica, hanno un diametro di circa 25-150 nm e sono stabilizzate dalla presenza di una proteina specifica chiamata caveolina-1.

Le caveole sono state isolate trattando le cellule con Triton X-100 all'1% e separando l'estratto cellulare su un gradiente di saccarosio. Le caveole sono state trovate associate alle frazioni a bassa densità di saccarosio, che contengono le frazioni di membrana resistenti al detergente in cui sono presenti molte proteine che comprendono anche la caveolina (Rothberg *et al.* 1992), le proteine legate alle GPI (Brown *et al.* 1992) e numerose proteine coinvolte nella trasduzione del segnale (Foster *et al.* 2003, Von Haller *et al.* 2001).

L'identificazione di proteine che partecipano a vie di trasduzione del segnale, in frazioni a bassa densità di saccarosio, ha fatto ipotizzare che i domini di membrana resistenti al Triton X-100 fossero coinvolti in diversi processi biologici.

Gli studi condotti da Schnitzer hanno dimostrato che, nelle frazioni a bassa densità di saccarosio, sono presenti due tipi di domini lipidici: le caveole, caratterizzate dalla presenza di caveolina, e i "lipid rafts", che non contengono caveolina ma solo le proteine legate alle GPI (Schnitzer *et al.* 2000).

I domini di membrana denominati "lipid rafts" sono ricchi in sfingolipidi e colesterolo, e sono implicati in molte vie di trasduzione del segnale come le vie che regolano l'apoptosi, l'adesione e la migrazione cellulare, la

trasmissione sinaptica, l'organizzazione del citoscheletro e il *sorting* delle proteine (Brown *et al.* 1998; Anderson *et al.* 1998; Tooze *et al.* 2001; Wolozin *et al.* 2001; Harris *et al.* 2002; Tsui-pierchala *et al.* 2002).

Esiste una correlazione biologica tra i “lipid rafts” e i segnali intracellulari mediati dal recettore RET?

Nei meccanismi di trasduzione del segnale mediati dal recettore RET, il co-recettore GFR α -1 è ancorato ai “lipid rafts” di membrana mediante il legame con le GPI. È stato dimostrato che in assenza di stimolazione, e quindi nella conformazione inattiva, RET è localizzato esclusivamente in zone della membrana in cui non sono presenti i “lipid rafts” mentre in seguito alla stimolazione con GDNF RET viene reclutato nei “lipid rafts” grazie al legame con il GFR α -1 (figura 6) per trasmettere i segnali intracellulari (Tansey *et al.* 2000). Gli stessi autori hanno anche dimostrato che impedendo la localizzazione di RET nei “lipid rafts” si ha un forte rallentamento della differenziazione e della sopravvivenza neuronale, anche se RET è cataliticamente attivo.

Un dato interessante circa l'attivazione di RET nei “lipid rafts” è stato ottenuto trattando cellule MG87-RET (che non esprimono GFR α -1) con GFR α -1 solubile e con GDNF (Paratcha *et al.* 2001). A seguito del trattamento con ligando e co-recettore, RET trasloca nei “lipid rafts” nella conformazione attiva. Questi dati suggeriscono che durante l'attivazione di RET in presenza di GFR α -1 solubile, il recettore può essere attivato fuori

dai “rafts” per essere reclutato di seguito in questi microdomini di membrana. RET attivato in *trans*, e quindi senza che GFR α -1 sia ancorato alla membrana, potrebbe essere reclutato nei “lipid rafts” grazie ad una interazione con target proteici intracellulari.

RET attivato in *cis* (in presenza del GFR α -1 ancorato alla membrana) ma anche in *trans* (in presenza del GFR α -1 solubile) quando è localizzato in “lipid rafts” segnala attraverso il legame alla proteina adattatrice FRS2, mentre fuori dai “lipid rafts” segnala attraverso una proteina adattatrice differente, SHC (figura 7). La presenza di recettore attivato sia all’interno dei “lipid rafts” che in altri domini della membrana fa ipotizzare che la diversa localizzazione di RET sulla membrana possa essere alla base dell’attivazione di vie di trasduzione del segnale differenti da parte del recettore.

Rimane comunque da stabilire se RET migri nei “lipid rafts” in risposta ad uno stimolo oppure se venga trattenuto in questi microdomini solo dopo essere entrato a far parte del complesso multiproteico di segnale (figura 8).

LE FOSFATASI

La maggior parte delle proteine che fanno parte delle vie di trasduzione del segnale è soggetta ad una modificazione covalente, ma reversibile, che consiste nell'aggiunta di gruppi fosfato a specifici residui amminiacidici della catena polipeptidica (fosforilazione). La reversibilità della modificazione è assicurata da fosfatasi che provvedono alla rimozione dei gruppi fosfato (defosforilazione). Queste modificazioni biochimiche sono fondamentali per la trasmissione di segnali all'interno della cellula. La fosforilazione induce una modificazione conformazionale nella struttura della proteina bersaglio, facendo sì che la proteina passi da uno stato "inattivo" ad uno "attivo" o viceversa. Questo passaggio di stato, in seguito all'aggiunta di un gruppo fosfato, regola l'attività di molte proteine che partecipano alla trasmissione dei segnali che regolano la crescita, il differenziamento e lo sviluppo cellulare. Nelle cellule esiste una "superfamiglia" di proteine con attività chinasi, che cioè catalizzano l'aggiunta di un gruppo fosfato a livello di specifici residui delle proteine target localizzate a valle nella via di trasduzione del segnale. I residui fosforilati possono essere serina, treonina o tirosina. Analogamente, esiste una "superfamiglia" di proteine con attività fosfatasi, che catalizzano la rimozione dei gruppi fosfato da residui di fosfo-serine, fosfo-treonine e fosfo-tirosine. Poiché *in vivo* l'attività biologica delle chinasi e delle fosfatasi è ovviamente dinamica, lo stato di fosforilazione delle proteine

bersaglio è modulata dall'azione combinata di queste due famiglie di enzimi con attività opposte. Ogni alterazione dall'equilibrio fra le due attività può causare provocare disturbi del controllo della proliferazione e differenziazione cellulare. E' quindi evidente che un disciplinato funzionamento delle proteine di ciascuna famiglia, chinasi e fosfatasi, giochi un ruolo fondamentale nel mantenimento della omeostasi cellulare. Mentre l'identificazione e la caratterizzazione delle proteine con attività chinasi è cominciata da molto tempo, l'attenzione degli studiosi verso le fosfatasi è più recente, motivo per il quale si sa poco sulle loro caratteristiche funzionali e strutturali.

La superfamiglia delle fosfotirosino-fosfatasi

Le fosfotirosino-fosfatasi (PTPs) costituiscono una superfamiglia di enzimi specifici (figura 9) che svolgono funzioni critiche in molte vie di traduzione del segnale. I membri della superfamiglia sono caratterizzati dalla presenza, nel dominio catalitico, di un *consensus motif* HCx₅R che permette di classificarli in due categorie, *classical* PTPs e *dual-specificity* PTPs (Tonks and Neel 2001). Le tirosino fosfatasi che catalizzano la reazione di defosforilazione unicamente di fosfo-tirosine (pTyr) fanno parte delle *classical* PTPs e possono contenere uno o due domini catalitici di circa 240-250 amminoacidi. Il *consensus motif* è rappresentato dalla sequenza HCSAGxGRxG (H= istidina, C= cisteina, S= serina, A= alanina, G=

glicina, R= arginina). Le *classical* PTPs vengono suddivise, sulla base delle sequenze che fiancheggiano il dominio catalitico, in fosfatasi transmembrana (RPTPs) e fosfatasi non transmembrana (non-TM). Le sequenze fiancheggianti svolgono funzioni regolatorie poiché rappresentano siti d'interazione per le proteine target e/o per proteine regolatorie. Per esempio, il segmento extracellulare delle PTPs transmembrana contiene siti di legame per ligandi regolatori, i quali possono regolarne l'attività catalitica in risposta a stimoli specifici. La fosfatasi transmembrana meglio caratterizzata è l'enzima CD45. Fra le fosfatasi non-transmembrana sono state meglio caratterizzati gli enzimi PTP1B (*Protein Tyrosine Phosphatase 1B*) e le fosfatasi Shp1 e Shp2 (rispettivamente *Src homology two-domain containing phosphatase 1* e *2*). Le fosfatasi che defosforilano non solo residui di fosfo-tirosina (pTyr), ma anche residui di fosfo-serina e fosfo-treonina (pSer/pThr) fanno parte delle *dual-specificity* PTPs. Il *consensus motif* di queste proteine è costituito dalla sequenza HCxxGxxR. A questa categoria appartengono le MAP chinasi-fosfatasi (MKPs) e i membri della famiglia cdc25.

Tutti i membri della superfamiglia PTP agiscono con lo stesso meccanismo catalitico: attacco nucleofilo tra il residuo di cisteina presente nel *consensus motif* e il gruppo fosfato legato allo specifico residuo amminoacidico delle proteine bersaglio.

La struttura delle tirosino fosfatasi Shp-1 e Shp-2.

Il gene umano della fosfatasi Shp-1 (prima chiamata PTP1C, SHP, HCP o SH-PTP1) è localizzato sul cromosoma 12 (12p13) (Plutzky *et al.* 1992; Matsuscita *et al.* 1999). Il gene *shp-1* comprende 17 esoni, ed è lungo circa 17kb (figura 10) (Wu *et al.* 2003). Il gene *shp-1* possiede due differenti promotori, la cui attività è tessuto specifica e che danno origine a due differenti isoforme della proteina. Le due isoforme della proteina differiscono fra loro per il codone di inizio utilizzato. I due differenti codoni di inizio sono localizzati negli esoni 1 e 2, rispettivamente (figura 10). Le due isoforme quindi differiscono per la sequenza amminoacidica all’N-terminale, MLSRG per l’isoforma 1 e MVR per l’isoforma 2. Le differenze nell’attività enzimatica tra le due isoforme sono invece irrilevanti (Plutzky *et al.* 1992; Walton *et al.* 1993). L’espressione delle due isoforme è tessuto specifica, in quanto il promotore 1, che è localizzato circa 7kb a monte del promotore 2, è attivo in tutte le cellule tranne che nelle linee ematopoietiche, mentre il promotore 2 è attivo esclusivamente in queste ultime. I residui amminoacidici 1-108 e 116-208, all’estremità N-terminale della catena polipeptidica, formano due *Src-homology-2 domain*, denominati rispettivamente domini N-SH2 e C-SH2. I residui amminoacidici 270-532 formano il dominio catalitico (figura 11).

Il gene umano della fosfatasi Shp-2 (prima chiamata SH-PTP2, PTP1D, SH-PTP3 e Syp) è localizzato anch’esso sul cromosoma 12 (12q24). La

fosfatasi Shp-2 è una proteina che viene espressa ubiquitariamente in vari tipi di cellule e di tessuti. I residui amminoacidici 6-101 e 112-211 formano, come i due domini all’N-terminale di Shp-1, due domini SH2 (*Src-homology-2 domains*), denominati anche in questo caso domini N-SH2 e C-SH2. La regione compresa fra i residui amminoacidici 276-519 contiene il dominio catalitico (figura 12). Le strutture tridimensionali delle fosfatasi Shp-1 e Shp-2 presentano un elevato livello di omologia e la determinazione della struttura cristallografica di Shp-1 rivela che i suoi tre domini sono assemblati come quelli di Shp-2 (figura 13). L’interazione tra il dominio N-SH2 e il dominio catalitico (PTP) suggerisce che sia Shp-1 che Shp-2 usino il dominio N-SH2 per mantenere l’enzima in una conformazione inattiva (Yang *et al.* 2003), e che la elevata mobilità del dominio C-SH2 funzioni come un’antenna per identificare i fosfopeptidi attivatori. Infatti, il legame di un fosfopeptide con il dominio C-SH2 comporta una modificazione conformazionale della molecola per cui il dominio N-SH2 si apre per interagire con un secondo fosfopeptide, liberando così il dominio catalitico e rendendolo pronto a catalizzare la reazione biologica (figura 14).

Il coinvolgimento delle tirosino fosfatasi Shp-1 e Shp-2 nei meccanismi delle trasduzione del segnale mediati da recettori di membrana

Molti recettori di membrana, ad esempio i recettori per i fattori di crescita, possiedono una attività tirosino chinastica intrinseca che viene attivata dal legame del recettore con una molecola di ligando che ne induce la dimerizzazione. Come conseguenza della dimerizzazione, le molecole di recettore si transfosforilano in tirosina e si attivano cataliticamente. Una volta attivato, il recettore lega le proteine bersaglio mediante il riconoscimento tra la tirosina fosforilata del recettore e il dominio SH2 o PTB della proteina bersaglio.

Le fosfatasi Shp-2 e Shp-1, contengono entrambi domini SH2 e vengono attivate dal legame del dominio SH2 con le fosfotirosine dei recettori di membrana, o con le fosfotirosine presenti su proteine citosoliche. Poichè Shp-1 ed Shp-2 sono localizzate, nella via di trasduzione del segnale, a valle dei recettori di membrana, in seguito alla loro attivazione si innescano complesse interazioni proteiche che trasmettono il segnale all'interno della cellula, allo scopo di guidarla verso la proliferazione o la differenziazione.

(A) Le vie di trasduzione del segnale nelle quali è specificamente coinvolta la tirosino fosfatasi Shp-2

La tirosino fosfatasi Shp-2 è espressa ubiquitariamente e si pensa che partecipi a diversi meccanismi di trasduzione del segnale, compresi quelli

che partono dalla stimolazione delle cellule con fattori di crescita come PDGF (*Platelet-derived growth factor*), EGF (*Epidermal growth factor*) ed IGF-1 (*insulin-like growth factor-1*), con citochine come IL-3, GM-CSF ed EPO, ed ancora con insulina ed interferone (Neel *et al.* 1997; Huyer *et al.* 1999).

La tirosina fosfatasi Shp-2 è definita un effettore positivo nella trasmissione di segnali all'interno della cellula in quanto partecipa sia all'attivazione della via Ras/MAPK che all'attivazione della via PI3K/AKT.

Fibroblasti stimolati con il fattore di crescita dell'epidermide, EGF, incrementano l'emivita della forma attivata di Ras chiamata GTP-Ras. La conversione della forma GTP-Ras (cataliticamente attiva) alla forma GDP-Ras (cataliticamente inattiva) è mediata da Ras-GAP che trasloca sulla membrana plasmatica perchè lega il recettore per l'EGF sulla tirosina fosforilata in posizione 992 (Agazie *et al.* 2003). L'ipotesi è che la fosfatasi Shp-2 ha un ruolo critico nell'aumento dell'emivita della forma GTP-Ras poichè, defosforilando la fosfo-tirosina 992 dell'EGFr, impedisce il legame Ras-GAP/EGFr che è alla base della conversione della forma GTP-Ras nella forma GDP-Ras.

Studi usando fibroblasti derivati da topi $Gab1^{-/-}$ e $FRS2^{-/-}$ hanno indicato che le interazioni tra le proteine adattatrici Gab1 ed FRS2 con la fosfatasi Shp-2 sono indispensabili per l'attivazione della via Ras/Erk mediata dalla

stimolazione delle cellule con i fattori di crescita PDGF e EGF (Itoh *et al.* 2000).

È stato dimostrato che, stimolando fibroblasti con il fattore di crescita FGF, la fosfatasi Shp-2 potrebbe defosforilare la proteina Sprouty (Spry) (Hanafusa *et al.* 2002). La proteina Spry è fosforilata in tirosina in seguito a stimolazione con il fattore di crescita FGF e come conseguenza della fosforilazione in tirosina di Spry si verifica l'inibizione della via di trasduzione del segnale Ras-MAP chinasi. L'ipotesi che Spry sia un substrato della fosfatasi Shp-2 è stata confermata in un recente lavoro in cui è stato dimostrato che la defosforilazione di Spry da parte di Shp-2 stabilizza l'attivazione della via Ras-MAP chinasi in risposta all'FGF (*fibroblast growth factor*) (Hanafusa *et al.* 2004).

Il fattore trascrizionale nucleare NF-kB ha un ruolo importante in molti processi infiammatori, ed è attivato dalla stimolazione delle cellule con fattori che includono il fattore alfa di necrosi tumorale, l'interleuchina-1 e i raggi UV. Molti lavori suggeriscono che l'attivazione del fattore trascrizionale NF-kB è mediato anche dall'attivazione del recettore per il fattore di crescita dell'EGF. Quello che è noto è che, stimolando con EGF cellule di glioma U87, l'attivazione del recettore dell'EGF provoca una interazione tra la fosfatasi Shp-2 e la proteina adattatrice Gab1. Il complesso Shp-2/Gab1 attiva la via PI3K/AKT e come conseguenza è attivato il fattore trascrizionale NF-kB (Kapoor *et al.* 2004).

La stimolazione di fibroblasti di ratto 3T3 con i fattori di crescita EGF e PDGF attiva la chinasi Src. Neel e colleghi hanno dimostrato che inibendo l'espressione della fosfatasi Shp-2 in fibroblasti 3T3, si verifica una iperfosforilazione della tirosina inibitoria 529 di Src. L'ipotesi è che a regolare i livelli di fosforilazione della tirosina inibitoria 529 di Src è proprio l'attività biologica della fosfatasi Shp-2 (Zhang *et al.* 2004).

È stato riportato che in motoneuroni di ratto MN1 la stimolazione di RET con il GDNF attiva la via PI3K/AKT attraverso la formazione di un complesso multiproteico che include RET, la fosfatasi Shp-2 e le proteine adattatrici SHC, Grb2 e Gab2 (Besset *et al.* 2000).

Questi dati, nel loro insieme, portano alla conclusione che la fosfatasi Shp-2 funziona generalmente come effettore positivo sia nella differenziazione che nella proliferazione cellulare.

(B) Vie di trasmissione del segnale a cui partecipa la tirosina fosfatasi Shp-1

Il dominio catalitico di Shp-1 può legare la sequenza consenso (D/E)X(L/I/V)X₁₋₂pYXX(L/I/V), definita il motivo ITIM (*immunoreceptor tyrosine-based inhibition motif*). Questo motivo strutturale può trovarsi in proteine differenti, molte delle quali sono recettori transmembrana per ligandi dotati di attività inibitoria.

Studi condotti su topi motheaten (*me*) e topi viable motheaten (*me^v*), ceppi mutanti spontanei che non esprimono la proteina Shp-1, hanno definito che la fosfatasi Shp-1 è importante per la maturazione e la funzione dei linfociti. Infatti, i topi omozigoti per *me* oppure *me^v* sviluppano malattie autoimmuni che ne causano la morte dopo 3-9 settimane. Una ridotta espressione di Shp-1 è stata riscontrata in molti casi di linfoma di Burkitt, conosciuti come linfoma umano delle cellule B (Delibrias *et al.* 1997).

Nei linfociti B, Shp-1 lega *motif* ITIM presente nei recettori inibitori, come CD22 e CD72, mediante il proprio dominio SH2 e defosforila le proteine a valle. Come conseguenza si può verificare o la terminazione di segnali attivatori oppure l'attivazione di altre vie come ad esempio la via apoptotica.

L'espressione della fosfatasi Shp-1 è notevolmente ridotta in molte linee di carcinoma e la crescita di cellule cancerose viene soppressa dopo l'introduzione del gene *shp-1* nelle corrispondenti linee cellulari. Shp-1 potrebbe quindi funzionare come un potente soppressore tumorale.

In cellule epiteliali maligne MCF7 (carcinoma della mammella), è stato dimostrato che Shp-1 è associata al recettore del PDGF (Vogel *et al.* 1993) ed alla subunità p85 di PI3K (Yu *et al.* 1998) attraverso il proprio dominio N-SH2. In cellule MCF7, la stimolazione con il PDGF riduce i livelli di attivazione del promotore di *c-fos*. Poiché in questa linea cellulare Shp-1 è

overespressa, si pensa che la fosfatasi regoli negativamente la via del recettore per il PDGF.

Il recettore per il fattore di crescita EGF è un importante induttore della motilità e della mitogenesi dei fibroblasti del derma. Durante l'invecchiamento cellulare, sia *in vivo* che *in vitro*, i fibroblasti dimostrano una ridotta capacità di migrare e di proliferare in risposta a fattori di crescita (Shiraha *et al.* 2000). Di recente è stato dimostrato che cellule Hs68 (fibroblasti umani) mostrano una ridotta fosforilazione in tirosina del recettore per l'EGF durante l'invecchiamento cellulare, e che questa diminuzione è correlata da un aumento nei livelli di espressione della fosfatasi Shp-1 (Kien *et al.* 2003).

Il fattore di crescita NGF (*nerve growth factor*) media la sopravvivenza e la differenziazione di cellule neuronali stimolando l'attività tirosinica del recettore TrkA. In cellule neuronali PC12, il recettore TrkA è a sua volta regolato negativamente dalla fosfatasi Shp-1 (Marsh *et al.* 2003). Shp-1 lega il recettore TrkA a livello del residuo di fosfo-tirosina 490 con il proprio dominio SH2. Il legame induce la defosforilazione delle fosfotirosine Y674/675 di TrkA, con conseguente induzione di apoptosi.

Studi condotti sul coinvolgimento della fosfatasi Shp-1 nei segnali intracellulari mediati da un mutante del recettore RET attivato costitutivamente (Ret9bp), hanno dimostrato che in cellule NIH3T3 la co-espressione stabile di Shp-1 e Ret9bp riduce di circa il 20% la

fosforilazione del recettore ed abbassa a circa il 40% la sua capacità trasformante con un decremento dell'attivazione di Erk (Hennige *et al.* 2001).

Considerati nell'insieme, questi risultati definiscono la fosfatasi Shp-1 come effettore negativo in più vie di trasduzione del segnale.

SCOPO DELLA TESI

Shp-1 e Shp-2 sono fosfatasi citosoliche coinvolte negli eventi di trasduzione del segnale mediati da recettori di membrana. Sebbene presentino il 55% di identità di sequenza, le due proteine svolgono funzioni biologiche opposte. Shp-1 è espressa soprattutto in cellule ematopoietiche e funziona come regolatore negativo in diversi sistemi cellulari, anche quelli non ematopoietici. Viceversa, Shp-2 è una proteina espressa ubiquitariamente che funziona come regolatore positivo in molte vie di trasduzione del segnale che partono da recettori tirosino chinasi e recettori di citochine.

Le mutazioni nel gene *ret*, che codifica per un recettore con attività tirosino chinasi, sono alla base di sindromi tumorali come la Neoplasia Endocrina Multipla di tipo 2 (MEN 2) e il Carcinoma Midollare Familiare delle Tiroide (FMTC). Entrambe le sindromi sono ereditate per via germinale.

Il coinvolgimento delle fosfatasi Shp-1 e Shp-2, nei meccanismi di trasduzione del segnale a valle del recettore RET non è conosciuto (Besset *et al.* 2000; Kurokawa *et al.* 2001; Hennige *et al.* 2001).

Lo scopo della tesi è stato quello di analizzare sia le interazioni di Shp-1 e Shp-2 con il recettore RET che l'effetto di tale interazione nei meccanismi di trasduzione del segnale mediati dai mutanti di RET (RET^{C634Y} e RET^{M918T}). I mutanti RET^{C634Y} e RET^{M918T} sono rispettivamente associati alle sindromi tumorali MEN2A e MEN2B.

Poiché sia Shp-2 che Shp-1 segnalano a valle di recettori di membrana, abbiamo valutato se la localizzazione del recettore RET in differenti microdomini di membrana potesse regolare in maniera differenziale il riconoscimento fra le due fosfatasi e la proteina RET.

Per questo studio abbiamo usato come sistema modello cellule di feocromocitoma di ratto PC12 trasfettate stabilmente con due differenti alleli mutanti del recettore RET (RET^{C634Y} e RET^{M918T}), che codificano molecole attivate costitutivamente. Le ragioni della scelta di questo sistema cellulare sono duplici: 1) i feocromocitomi sono tumori caratteristici della patologia MEN2 e quindi le PC12 sono cellule molto simili alle linee cellulari che esprimono RET in condizioni fisiologiche; 2) le cellule esprimono sia Shp-1 che Shp-2.

I risultati ottenuti sono stati oggetto di pubblicazione (D'alessio *et al.* 2003; Incoronato *et al.* 2004; Amoresano *et al.* 2004; Chalet-Berguerand *et al.* 2004)

MATERIALI E METODI

1. Colture cellulari e trasfezioni

La linea cellulare PC12 è stata cresciuta in mezzo RPMI 1640 (GIBCO) addizionato con 10% siero di cavallo, 5% siero fetale bovino e 2mM L-glutammina. Le linee cellulari PC12/Ret^{C634Y}, PC12/Ret^{M918T} e PC12/Ret^{wt}, sono state ottenute trasfettando stabilmente la linea parentale PC12 con i plasmidi di espressione in eucarioti in cui è stato clonato il cDNA corrispondente a RET^{C634Y}, RET^{M918T} e RET^{wt} umano nella isoforma Ret9, rispettivamente. Le linee cellulari sono state selezionate per la capacità di crescere in presenza di 0,025mg/ml acido micofenolico (SIGMA), e selezionate per tre settimane in un mezzo di selezione *gpt* contenente 0,25mg/ml di Xantina (SIGMA) addizionato con sali HAT (SIGMA).

La linea cellulare PC12- α 1/wt è stata ottenuta trasfettando stabilmente cellule PC12/Ret^{wt} con un plasmide di espressione in eucarioti in cui è stato clonato il cDNA corrispondente al GFR α -1, e selezionata per tre settimane in un mezzo selettivo *gpt* in presenza di 0,4mg/ml di G418.

Le linee cellulari PC12/RetC634Y^{Shp-2wt} e PC12/RetM918T^{Shp-2(c/s)} sono state ottenute trasfettando stabilmente un plasmide di espressione in cui è stato clonato il cDNA di myc-Shp-1 wt e di myc-Shp-2(c/s), e selezionate per tre settimane in un mezzo di selezione *gpt* in presenza di 0,4mg/ml di G418.

Tutti gli esperimenti di trasfezione cellulare sono stati eseguiti usando come reagente la Lipofectamina 2000 (Invitrogen) seguendo le istruzioni della ditta. Per le trasfezioni transienti le cellule, 24 h dopo che sono state piastrate, sono state trasfettate con 0,5µg i plasmidi d'espressione per i mutanti di RET (RET^{C634Y} e RET^{M918T}) in presenza di 2µg del plasmide reporter. Il plasmide pEBG-mBad contiene la sequenza relativa al gene Bad fuso al gene glutatione-S-transferasi (GST) (New England Biolabs, Inc., Beverly, MA). Il plasmide pCEFL-HA-Akt contiene il gene per la proteina Akt fuso all'epitopo emagglutinina (HA). Dove è indicato, le cellule sono state stimulate aggiungendo nel mezzo di crescita 100ng/ml GDNF (Promega) oppure 100ng/ml 2.5S NGF (Upstate Biotechnology, Lake Plaid, NY).

2. Clonaggio

La proteina di fusione GST-(N+C SH2)Shp-1, che codifica gli amminoacidi 1-251 della proteina Shp-1, è stata generata per PCR (*polimerase chain reaction*) dal cDNA di Shp-1 usando i primer 5'-ATGAGATCTATGCTGTCCCGTGGGTGG-3' e 5'-CATGAATTCTTACTCAAACCTCCCAGAA-3'. Il prodotto ottenuto per PCR è stato digerito con gli enzimi di restrizione EcoRI e BglII ed è stato legato al vettore pGEX-4T3. La proteina GST-(N+C SH2)Shp-1 è

stata ottenuta trasformando il batterio *Escherichia coli* con il plasmide pGEX-4T3/(N+C SH2)Shp-1. I batteri trasformati con il plasmide sono stati cresciuti alla temperatura di 37°C fino ad un valore di assorbimento a 600nm pari a 0,6 OD e sono stati stimolati con 1mM di IPTG per 3 h. La proteina GST-(N+C SH2)Shp-1 è stata purificata ad omogeneità su cromatografia di affinità usando la resina glutatione-sefariosio. Il plasmide GST-(N+C SH2)Shp-2 c'è stato gentilmente fornito da B. Neel.

3. Preparazione di estratti cellulari, immunoprecipitazioni e immunoblotting

Per la preparazione degli estratti cellulari, le cellule sono state lavate in PBS freddo e lisate in buffer A, 50mM Tris-HCl pH 8.0 contenente 150mM NaCl, 1% Nonidet P-40, 2 µg/ml aprotina, 1 µg/ml pepstatina, 2 µg/ml leupeptina e 1 mM Na₂VO₄. La concentrazione proteica dell'estratto cellulare è stata determinata usando il dosaggio Bradford usando come standard l'albumina di siero bovino.

Per gli esperimenti di immunoprecipitazione, gli estratti cellulari (1 mg) sono stati incubati per 2 h alla temperatura di 4°C con anticorpi specifici e quindi immunoprecipitati con la proteina G coniugata all'agarosio (G-plus agarosio, Calbiochem, Oncogene Research) per 16 h a 4°C. Gli immunoprecipitati sono stati lavati 5 volte con il buffer A, denaturati in

buffer Laemmli per 5' a 100°C e caricati su un gel SDS-PAGE (8% acrilammide). Le proteine sono state elettroblottate su una membrana PVDF (Millipore, Bedford, MA), immunoblottate con specifici anticorpi primari e anticorpi secondari coniugati con la perossidasi, ed analizzate usando un sistema di chemiluminescenza ECL (Amersham-Pharmacia Biosciences).

Gli anticorpi primari che abbiamo usato sono: anti-RET (H-300), anti-Shp-1 (C-19), anti-Shp-2 (C-18), anti-Gab2, anti-myc (9E10) della Santa Cruz Biotechnology, CA; anti-fosfo-tirosina (4G10) della Upstate Biotechnology; anti-GST della Amersham Pharmacia Biosciences; anti-HA della Roche Molecular Biochemicals; anti-pAkt (Ser473), anti-Bad, anti-Bad-112 e anti-Bad-136 della New England Biolabs Inc.

Quando è indicato, i filtri sono stati strippati in 65,5 mM Tris-HCl pH 6,7 in presenza di 100 mM 2-mercaptoetanolo e 2% sodio dodecil solfato (SDS) per 30 minuti a 55°C.

Per gli immunoblott mostrati in figura, sono state eseguite analisi statistiche usando il programma per PC, NIH image.

4. Gradienti di Saccarosio

Le cellule PC12/Ret^{C634Y}, PC12/Ret^{M918T} e PC12- α 1/wt, sono state lavate in PBS freddo e lisate in 2 ml di buffer B, 20 mM tris-HCl pH 8.0 contenente

15 mM NaCl, 1% Triton X-100, 10 mM EDTA, 2 µg/ml aprotinina, 1 µg/ml pepstatina, 2 µg/ml leupeptina e 1 mM Na₂VO₄. Ai 2 ml di lisato cellulare, sono stati aggiunti 2ml di 80% saccarosio ottenendo una soluzione al 40% saccarosio. I 4ml al 40% saccarosio, contenenti i lisati cellulari, sono stati posti sul fondo di un tubo per centrifuga da 12 ml e uno step di gradiente di saccarosio (5-35% saccarosio sciolto in 20mM Tris-HCl pH 7,5, 15 mM NaCl e 10 mM EDTA) è stato adagiato sui 4 ml di saccarosio al 40%. I campioni sono stati centrifugati a 260 000 x g per 16 h in un rotore SW41 della Beckman (Fullerton, CA) e raccolti in frazioni da 1 ml. Le proteine contenute nelle singole frazioni sono state precipitate in 20% acido tricloro acetico (TCA), solubilizzate e denaturate in buffer Laemmli per 5 minuti a 100°C. I campioni così preparati sono stati caricati su un gel SDS-PAGE (8% acrilammide) ed immunoblottati con specifici anticorpi. Dove è indicato, le singole frazioni del gradiente di saccarosio sono state immunoprecipitate con anticorpi anti-Shp-1 o anti-Shp-2.

5. Analisi per “Far Western Blotting “ e dosaggi “GST pull-down”

In esperimenti di Far Western Blotting, 200µg di estratti cellulari sono stati caricati su un SDS-PAGE (8% acrilammide). Le proteine sono state elettroblottate su membrana PVDF e il filtro è stato incubato, per 1 h a temperatura ambiente, in 20mM Tris-HCl pH 7,5 contenente 15mM NaCl,

0,1% Tween 20 e 5% latte non grasso. Dopo l'incubazione il filtro è stato ibridato, per 30 minuti a temperatura ambiente, con 1,5 µg/ml della proteina di fusione GST-(N+C SH2)-Shp-1 oppure della proteina di fusione GST-(N+C SH2)-Shp-2. Per identificare il legame tra la proteina fusa alla GST e le proteine fosforilate in tirosina, il filtro è stato immunoblottato con anticorpi anti-GST.

Nei dosaggi GST pull-down, 1 mg di estratto cellulare è stato incubato, per 1 h a 4°C, con 30 µl di resina glutatione sefarosio e 50 µg della proteina fusa alla GST. La resina è stata lavata in buffer B e sospesa in 30 µl di buffer Laemmli. Le proteine legate alla resina sono state denaturate per 5 minuti a 100°C, caricate su SDS-PAGE (8% acrilammide) ed immunoblottate usando anticorpi anti-Ret (H300).

6. Dosaggi di fosfatasi

Un milligrammo di estratto grezzo di cellule PC12/Ret^{C634Y} e PC12/Ret^{M918T} è stato immunoprecipitato per 2 h a 4°C con anticorpi anti-Shp-1 o anti-Shp-2 in presenza della proteina G coniugata all'agarosio. Dopo l'incubazione la resina è stata lavata prima con buffer B e poi con 50mM HEPES contenente 5mM EDTA e 10mM DTT (buffer C). Gli immunoprecipitati sono stati incubati in una miscela di reazione [buffer C in presenza di 20nM microcisteina-leucina-arginina (Alomone

Laboratoires) e 10 μM ZnCl_2] alla temperatura di 30°C fino ad un tempo di 30 minuti. La reazione è stata bloccata aggiungendo 20 μl di buffer Laemmli 3X. Dopo la denaturazione i campioni sono stati caricati su 8% SDS-PAGE, le proteine sono state trasferite su membrana PVDF ed immunoblottate con anticarpi anti-pTyr.

7. Identificazione del DNA frammentato e dosaggi TUNEL

Per l'estrazione del DNA frammentato, $2,5 \times 10^6$ cellule sono state incubate per 16 h e 40 h in un mezzo di crescita privo di siero, quindi lisate in buffer 5mM Tris-HCl pH 7.4 in presenza di 0,5 % Triton X-100 e 20mM EDTA. I nuclei intatti sono stati rimossi mediante centrifugazione e il DNA solubile è stato purificato con estrazione fenolo/cloroformio e precipitato in 75% etanolo. Il DNA frammentato è stato analizzato mediante elettroforesi su gel 1,2% agarosio.

Per i dosaggi TUNEL, $2,5 \times 10^6$ cellule sono state trattate come descritto dalle specifiche del Kit Roche Molecular Biochemicals. L'apoptosi è stata identificata usando il colorante Fast Red (Dako Co., Carpenteria, CA).

8. Dosaggi CAT

Gli estratti cellulari sono stati prelevati dopo 72 h dalla trasfezione e l'attività della proteina cloramfenicolo-acetil-transferasi (CAT) è stata

analizzata mediante cromatografia su strato sottile come è descritto da Califano e colleghi (Califano *et al.* 1996). Gli spots sono stati isolati individualmente e contati allo scintillatore liquido. Per ciascun esperimento, la percentuale di conversione a cloramfenicolo-acetilato- C^{14} è stato calcolato e normalizzato per l'efficienza di trasfezione. La stessa efficienza di trasfezione è stata confermata con dosaggi di luciferasi, trasfettando le cellule con il reporter plasmidico pSV-Luc.

9. Dosaggi di chinasi per la proteina AKT

Cellule PC12 sono state trasfettate transientemente con i plasmidi d'espressione pMV7-Ret^{C634Y}, pMV7-Ret^{M918T}, pCEFL-HA-Akt, e pcDNA3.1Shp-2(c/s) ad un rapporto molare Ret/Akt = 1/6 e Ret/Shp-2 = 1/6. Le cellule sono state raccolte 48 h dopo la trasfezione, lisate con buffer B e 0.5 mg dell'estratto grezzo sono stati immunoprecipitati con anticorpi anti-HA per 2 h a 4°C. I campioni sono stati lavati in un buffer di reazione 20mM HEPES pH 7,4 contenente 10mM MgCl₂ e 10mM MnCl₂ ed incubati nello stesso buffer in presenza di 60 µg/ml di istone 2B (Upstate Biotechnology), 1mM ATP, 1mM DTT e 10µCi di [α -³²P]ATP per 30 minuti a 25°C, la reazione è stata bloccata aggiungendo buffer Laemmli. I prodotti della reazione della chinasi sono stati caricati su 8% SDS-PAGE, trasferiti su membrana PVDF ed autoradiografati.

RISULTATI

1. Shp-1 e Shp-2 legano il recettore di membrana RET formando due complessi multiproteici distinti.

E' noto che le fosfatasi Shp-2 e Shp-1 possono partecipare alle vie di trasduzione del segnale mediate da RET, ma non è ancora chiaro se leghino RET direttamente, nè quali siano le loro proteine bersaglio.

Poiché entrambe le fosfatasi sono localizzate a valle di recettori di membrana, ci siamo chiesti se, in un sistema cellulare in cui il recettore RET è attivo costitutivamente, Shp-1 e Shp-2 possano far parte di un complesso multiproteico in cui è presente RET. A tale scopo, abbiamo usato cellule PC12 trasfettate stabilmente con RET^{wt}, oppure con gli alleli mutati RET^{C634Y} e RET^{M918T}, rispettivamente cellule PC12/wt, PC12/Ret^{C634Y} e PC12/Ret^{M918T}. Queste linee cellulari esprimono livelli comparabili del recettore (Califano *et al.* 2000). Le colture cellulari sono state lisate come descritto in Materiali e Metodi e ciascun lisato cellulare è stato immunoprecipitato usando anticorpi anti-Shp-1 (figura 15 A, destra) oppure anticorpi anti Shp-2 (A, sinistra) ed è stato immunoblottato usando anticorpi anti-pTyr (pannello *a*) e anti-RET (pannello *b*). Come è mostrato in figura, le proteine RET mutate (RET^{C634Y} nella linea cellulare PC12/Ret^{C634Y} e RET^{M918T} nella linea cellulare PC12/Ret^{M918T}) co-immunoprecipitano con ciascuna fosfatasi (pannelli *a* e *b*, canali 3, 4, 7 e

8), viceversa la proteina RET non mutata (RET^{wt} nella linea cellulare PC12/wt) non co-immunoprecipitata con nessuna delle due fosfatasi (pannelli *a* e *b*, canali 1 e 2). Allo scopo di verificare se le due fosfatasi fanno parte dello stesso complesso multiproteico, il filtro è stato immunoblottato con anticorpi anti-Shp-1 (pannello *d*) e anti-Shp-2 (pannello *c*). Come è riportato in figura, negli immunoprecipitati con anticorpi anti-Shp-1, la fosfatasi Shp-1 non co-immunoprecipita con la fosfatasi Shp-2 (pannello *c*, canali 1 a 4) e negli immunoprecipitati con anticorpi anti-Shp-2, la fosfatasi Shp-2 non co-immunoprecipita con la fosfatasi Shp-1 (pannello *d*, canali da 5 a 8). Ciò suggerisce che i due complessi sono distinti e che ciascuna fosfatasi può legare le forme mutate di RET ma non l'altra fosfatasi. Per confermare che le due fosfatasi non coesistono nello stesso complesso multiproteico, abbiamo eseguito due ulteriori passaggi di immunoprecipitazione. Per deprivare i sopranatanti, immunoprecipitati con anticorpi anti-Shp-1 o anticorpi anti-Shp-2, dai complessi Shp-1/RET e Shp-2/RET, rispettivamente, abbiamo immunoprecipitato ciascun supernatante per la seconda volta con lo stesso anticorpo (B). In seguito, i sopranatanti privi dei complessi Shp-1/RET sono stati immunoprecipitati con anticorpi anti-Shp-2 (C, sinistra) e i sopranatanti privi dei complessi Shp-2/RET sono stati immunoprecipitati con anticorpi anti-Shp-1 (C, destra). Come è mostrato nella figura, nei sopranatanti deprivati dei complessi Shp-1/RET sono presenti i complessi

Shp-2/RET (primo pannello canali 3 e 4) e nei sopranatanti deprivati dei complessi Shp-2/RET sono presenti i complessi Shp-1/RET (primo pannello canali 7 e 8). Questi risultati indicano che, in un sistema cellulare in cui RET è attivo costitutivamente, il recettore media la formazione di due distinti complessi multiproteici, uno in cui il recettore RET è legato alla fosfatasi Shp-1 e l'altro in cui il recettore è legato alla fosfatasi Shp-2. RET^{wt} in assenza di stimolazione con il GDNF non è in grado di formare complessi con nessuna delle due fosfatasi (C, canali 2 e 6).

2. La localizzazione nei microdomini di membrana “lipid rafts” delle proteine RET mutate e delle fosfatasi Shp-1 e Shp-2.

L'attivazione del recettore RET, mediato dal legame del recettore al fattore di crescita GDNF e al co-recettore GFR α -1, induce la traslocazione del recettore in microdomini di membrana chiamati “lipid rafts” (Tansey *et al.* 2000; Paratcha *et al.* 2001). Abbiamo analizzato la distribuzione di membrana dei mutanti di RET (RET^{C634Y} e RET^{M918T}) e delle proteine Shp-1 e Shp-2 usando dei gradienti di saccarosio all'equilibrio di densità. Come descritto in Materiali e Metodi, le singole frazioni dei gradienti sono stati precipitati in 20% TCA, e le proteine presenti nelle frazioni sono state analizzate usando anticorpi anti-RET, anti-Shp-1, anti-Shp-2 e anti-pTyr (figura 16, A e B). Come è mostrato in figura, una significativa quantità di entrambe le forme mutante di RET, circa il 7,6% di RET^{C634Y} (A, sinistra

pannello *a*, e C) e circa l'8,0% di RET^{M918T} (A, destra pannello *a* e C), sono state ritrovata nei "lipid rafts" (frazioni 4, 5 e 6). In assenza di stimolazione con GDNF, RET^{wt} non localizza nei "lipid rafts" (B, pannello *a* e C). Analizzando la distribuzione della fosfatasi Shp-1 nelle frazione del gradiente di saccarosio si nota che, nella linea cellulare PC12- α 1/wt in cui RET non è attivato, circa il 20% di Shp-1 si trova nelle frazioni del gradiente contenenti i "lipid rafts", mentre il restante 80% è presente nelle frazioni che non contengono "lipid rafts" (B, pannello *b* e D). Viceversa, nelle linee cellulari PC12/Ret^{C634Y} e PC12/Ret^{M918T} la fosfatasi Shp-1 è quasi tutta presente nelle frazioni che non contengono i "lipid rafts" (frazioni 8-12, A pannelli *b* e D). Analizzando la distribuzione della fosfatasi Shp-2 nelle frazione del gradiente di saccarosio si osserva che la fosfatasi è equamente distribuita lungo tutte le frazioni del gradiente, sia nella linea cellulare PC12/Ret^{C634Y} che nella linea cellulare PC12/Ret^{M918T} (A, pannelli *c*), mentre nella linea cellulare PC12- α 1/wt la fosfatasi Shp-2 è soprattutto localizzata nelle frazioni che non contengono i "lipid rafts" (B, pannello *c*). Da questi risultati si evince che l'attivazione costitutiva del recettore RET fa variare la distribuzione delle tirosino fosfatasi Shp-1 e Shp-2 nelle frazioni del gradiente. Infatti, nella linea cellulare in cui RET non è attivo (PC12- α 1/wt), Shp-1 localizza equamente in tutte le frazioni del gradiente mentre Shp-2 localizza soprattutto in domini di membrana che escludono i "lipid rafts". Viceversa, nelle linee cellulari in cui RET è

attivato (PC12/Ret^{C634Y} e PC12/Ret^{M918T}), Shp-1 è presente nelle frazioni che escludono i “lipid rafts” mentre Shp-2 è presente sia nelle frazioni che includono i “lipid rafts” che nelle frazioni che li escludono.

3. I complessi multiproteici Shp-1/RET ed Shp-2/RET attivati sono localizzati in differenti domini della membrana plasmatica.

Come abbiamo dimostrato precedentemente, RET attivato può localizzare sia all'interno che esternamente ai “lipid rafts” e questa diversa localizzazione di membrana può guidare l'interazione di RET con diverse proteine bersaglio. Dai risultati mostrati nella figura 16, sebbene in percentuale diversa, sia RET attivato che entrambe le fosfatasi possono localizzare nei “lipid rafts”. Poiché i mutanti di RET (RET^{C634Y} e RET^{M918T}) co-immunoprecipitano con Shp-1 e con Shp-2, ma le due fosfatasi non fanno parte dello stesso complesso multiproteico (figura 15), ci siamo chiesti se ciascun complesso potesse essere localizzato in differenti domini della membrana plasmatica.

A tale scopo, per verificare in quali frazioni del gradiente fossero presenti i complessi multiproteici Shp-1/RET e Shp2/RET, abbiamo eseguito esperimenti di co-immunoprecipitazione. Come mostrato nella figura 17, abbiamo frazionato su gradienti di saccarosio i lisati cellulari delle cellule PC12/Ret^{C634Y} (A) e PC12/Ret^{M918T} (B). Quindi, immunoprecipitando con anticorpi anti-Shp-1 le frazioni del gradiente, abbiamo dimostrato che la

fosfatasi Shp-1 co-immunoprecipita con le isoforme di RET attivato sia nelle frazioni che contengono i “lipid rafts” (frazioni 4 a 6) che nelle frazioni che escludono i “lipid rafts” (frazioni 9-12 riunite) (A e B pannelli di sinistra). Inoltre, poiché nelle frazioni che escludono i “lipid rafts” il segnale della proteina Shp-1 è a saturazione, abbiamo effettuato una serie di diluizioni della frazione 9-12. Come è mostrato in A e in B, comparando la quantità di Shp-1 nella frazione 6 (frazione che include i lipid rafts) con la quantità di Shp-1 nella diluizione 1/50 (frazione che esclude i “lipid rafts”), il legame di Shp-1 a ambedue le forme attivate di RET è nettamente favorito all’interno dei “lipid rafts” rispetto alle frazioni che escludono i “lipid rafts”. Riportando in un istogramma la quantità del recettore RET mutato che co-immunoprecipita con Shp-1 (C) si osserva che, il legame nei “lipid rafts” di Shp-1/RET^{C634Y} è decisamente favorito rispetto al legame Shp-1/RET^{M918T} (circa il 69% del totale in PC12/Ret^{C634Y} contro il 13.7% in PC12/Ret^{M918T}). Sembra plausibile che l’arricchimento preferenziale di Shp-1/RET^{C634Y} nei “rafts” dipenda dalla specifica conformazione del recettore che, sebbene mutato, deve comunque dimerizzare per essere attivo, viceversa il mutante RET^{M918T} è attivo come omodimero. Questa possibilità è incoraggiata dalla differente partizione, nelle frazioni del gradiente di saccarosio, delle molecole di RET fosforilate in tirosina (figura 16 A pannelli d). Infatti, le molecole di RET^{C634Y} fosforilate in tirosina si

accumulano nei “lipid rafts” mentre le molecole di RET^{M918T} fosforilate in tirosina si distribuiscono lungo tutto il gradiente di saccarosio.

Questa distribuzione è specifica per i complessi Shp-1/RET infatti, immunoprecipitando con anticorpi anti-Shp-2 le frazioni del gradiente di saccarosio contenenti le proteine delle cellule PC12/Ret^{C634Y} e PC12/Ret^{M918T}, sia il recettore RET^{C634Y} che il recettore RET^{M918T} co-immunoprecipitano con la fosfatasi Shp-2 esclusivamente nelle frazioni che escludono i “lipid rafts” (A e B pannelli di destra).

Questi risultati dimostrano che le due fosfatasi non fanno parte dello stesso complesso proteico in cui è presente il recettore RET attivato (figura 15) perchè il complesso Shp-1/RET attivato è localizzato in domini di membrana che includono i “lipid rafts” mentre il complesso Shp-2/RET attivato è localizzato esclusivamente in domini di membrana che escludono i “lipid rafts”.

4. Nelle linee cellulari PC12/ Ret^{C634Y} e PC12/ Ret^{M918T} le fosfatasi Shp-1 e Shp-2 legano proteine differenti

Allo scopo di determinare quali sono le proteine che legano Shp-1 e Shp-2 a seguito dell'attivazione dell'oncogene RET, abbiamo costruito delle proteine di fusione che contengono i domini SH2, di Shp-1 e Shp-2, fusi alla proteina glutatione-S-transferasi (GST). Queste proteine di fusione sono state utilizzate, in esperimenti di “Far Western Blotting” (vedi

Materiali e Metodi), come sonde per identificare le proteine fosforilate in tirosina nei lisati cellulari delle linee PC12/ Ret^{C634Y} e PC12/ Ret^{M918T} (figura 18 A). Poichè molte proteine, indipendentemente dalla presenza delle forme attivate di RET, sono fosforilate in tirosina dalla presenza del siero nel mezzo di crescita, abbiamo incluso nello stesso esperimento lisati cellulari di PC12/ Ret^{C634Y} e PC12/ Ret^{M918T} ottenuti crescendo le cellule sia in assenza che in presenza di siero. Come è mostrato in A nel pannello di sinistra, in cellule PC12/Ret^{C634Y} e ancora di più in cellule PC12/Ret^{M918T}, la proteina di fusione GST-(N+C SH2)Shp-1 riconosce una banda di circa 97kDa in peso (comparare canale 1 con canali 4 e 6). La proteina di 97kDa, riconosciuta dalla proteina di fusione GST-SH2/Shp-1, non è riconosciuta nei lisati di cellule PC12- α 1/wt sia in presenza che in assenza di stimolazione con GDNF (canali 1 e 2, rispettivamente). Inoltre, nelle stesse linee cellulari, la proteina di fusione GST-(N+C SH2)Shp-1 identifica debolmente una doppia banda con un peso di circa 180 kDa compatibile con il peso molecolare del recettore RET (comparare canali 1 con 2, 3, 4 e 6 rispettivamente). In parallelo, abbiamo eseguito gli stessi esperimenti usando la proteina di fusione GST-(N+C SH2)Shp-2. Come è mostrato nella figura 18 A (pannello centrale), la proteina di fusione GST-(N+C SH2)Shp-2 ibrida con un notevole numero di proteine fosforilate in tirosina tra cui anche la proteina da 97kDa riconosciuta dalla proteina di fusione GST-(N+C SH2)Shp-1. La proteina di 97kDa è compatibile con Gab2,

mentre l'identità delle altre proteine è del tutto sconosciuta. Inoltre, solo negli estratti citosolici di cellule PC12/Ret^{M918T}, la proteina di fusione GST-(N+C SH2)Shp-2 riconosce due proteine di circa 170-180kDa che potrebbero corrispondere a RET (A, canale 6 e pannelli inferiori). Come è dimostrato da esperimenti di “pull down” (vedi di seguito), il legame tra la fosfatasi Shp-2 e RET^{M918T} non è diretto indicando che le due proteine non sono identificabili con RET. Come controllo positivo dell'esperimento, abbiamo usato la proteina di fusione GST-Shc/FL (A, pannello di destra), poiché è noto che la proteina adattatrice SHC lega direttamente il recettore RET. Questi risultati indicano che, quando RET è attivato, le fosfatasi Shp-1 e Shp-2, legano attraverso i loro domini SH2, dei bersagli proteici differenti e che il legame tra entrambe le fosfatasi e i mutanti di RET non è diretto. Allo scopo di confermare questa ipotesi, abbiamo eseguito dosaggi di pull-down con le stesse proteine di fusione (vedi Materiali e Metodi). Questo metodo ha come scopo quello di determinare il riconoscimento diretto e specifico tra il dominio SH2 della proteina fusa alla GST e un dominio fosforilato in tirosina della proteina bersaglio. Come è mostrato nella figura 18 pannello B, le proteine di fusione GST-(N+C SH2)Shp-1 (canali 5-8) e GST-(N+C SH2)Shp-2 (canali 9-12) non legano né RET^{wt} stimolato con GDNF né i mutanti di RET. Come controllo positivo abbiamo utilizzato la proteina di fusione GST-Shc/FL (canali 9-12). Considerati nell'insieme, questi risultati indicano che entrambe le fosfatasi

interagiscono con il recettore RET e che il legame è mediato da proteine differenti.

5. RET attivato non è substrato delle fosfatasi Shp-1 e Shp-2

Sebbene le fosfatasi Shp-1 e Shp-2 sono legate al recettore RET attraverso degli intermedi proteici, non si può escludere l'ipotesi che il recettore possa essere un substrato delle due fosfatasi. A tale scopo, abbiamo eseguito dei dosaggi di fosfatasi *in vitro*. I lisati cellulari di cellule PC12/Ret^{C634Y} e PC12/Ret^{M918T} sono stati immunoprecipitati con anticorpi anti-Shp-1 e anticorpi anti-Shp-2 e il prodotto dell'immunoprecipitato è stato incubato a tempi crescenti in un buffer di reazione per le fosfatasi (figura 19 A e B). Ciascun immunoprecipitato è stato analizzato usando anticorpi anti-pTyr. Come è mostrato nel pannello A, negli immunoprecipitati con anticorpi anti-Shp-1, la fosforilazione in tirosina delle proteine mutante di RET (RET^{C634Y} e RET^{M918T}) non subisce decrementi durante i 15 minuti di incubazione. Come controllo interno, la freccia indica una proteina fosforilata in tirosina, con un peso di circa 97kDa, che è rapidamente defosforilata durante il primo minuto di incubazione. Allo stesso modo, negli immunoprecipitati usando anticorpi anti-Shp-2, la fosforilazione in tirosina delle proteine mutante di RET è costante fino a 10 minuti di incubazione (B). Come controllo interno, una proteina di circa 80-85kDa in peso è defosforilata durante i primi minuti di reazione (freccia).

I risultati ottenuti dai dosaggi di “pulldown” (figura 18) e dai dosaggi di fosfatasi *in vitro* (figura 19) indicano che i mutanti di RET non legano direttamente né la fosfatasi Shp-1 né la fosfatasi Shp-2, inoltre nessuno dei due mutanti è un substrato delle due fosfatasi.

6. La proteina adattatrice Gab2 fa parte del complesso multiproteico Shp-2/RET attivato

Come è stato dimostrato in esperimenti di Far western Blotting e di pull-down (figura 18), il legame tra il recettore RET attivato con la fosfatasi Shp-2 è probabilmente mediato da intermedi proteici. Un lavoro pubblicato da Besset e colleghi ha dimostrato che in motoneuroni MN1, la stimolazione di RET^{wt} con il fattore di crescita GDNF, media la formazione del complesso proteico Gab2/Shp-2 (Besset *et al.* 2000).

Basandoci su queste osservazioni, ci siamo chiesti se il legame tra la fosfatasi Shp-2 e il recettore RET mutato potesse essere mediato dalla proteina di ancoraggio Gab2. A tale scopo, i lisati cellulari di cellule PC12/Ret^{wt}, PC12/Ret^{C634Y} e PC12/Ret^{M918T}, sono stati immunoprecipitati con anticorpi anti-Gab2 ed analizzati mediante immunoblotting con anticorpi anti-pTyr, anti-RET, anti-Gab2 ed anti-Shp-2 (figura 20 A). Come è mostrato nel pannello di sinistra, ibridando il filtro con anticorpi anti-pTyr, co-immunoprecipitano con Gab2 diverse specie proteiche fosforilate in tirosina. Per verificare se ciascuno di questi complessi potesse

contenere il recettore RET attivato e la fosfatasi Shp-2, il filtro è stato tagliato ed ibridato con anticorpi anti-RET, anti-Gab2 e anti-Shp-2 (pannello di destra). Come è mostrato in figura, sia i mutanti attivi di RET (RET^{C634Y} e RET^{M918T}) che la fosfatasi Shp-2 (canali 2 e 3) co-immunoprecipitano con Gab2. RET^{wt} non stimolato con GDNF non co-immunoprecipita né con Shp-2 né con Gab2 (canale 1). Per confermare il risultato, i lisati delle stesse linee cellulari sono stati immunoprecipitati con anticorpi anti-Shp-2 (B, pannello di sinistra) e anticorpi anti-RET (B, pannello centrale). In entrambi gli immunoprecipitati la fosfatasi Shp-2 co-immunoprecipita con il recettore RET attivato. Inoltre, i mutanti attivi di RET co-immunoprecipitano anche con la subunità regolatoria p85 di PI3K (B pannello di destra). Consistentemente con i precedenti lavori e con i nostri precedenti risultati, questo esperimento suggerisce che in cellule PC12 RET attivato costitutivamente lega la fosfatasi Shp-2 attraverso la proteina di ancoraggio Gab2.

7. La tirosina fosfatasi Shp-2 è coinvolta nella differenziazione di cellule PC12 indotta dai mutanti di RET

A questo punto ci siamo chiesti quale potesse essere la risposta funzionale all'interazione della fosfatasi Shp-2 con ciascun mutante del recettore RET (RET^{C634Y} e RET^{M918T}). I mutanti del recettore RET inducono la trascrizione di geni ad espressione immediata, come *Egr-1* e *c-fos*, in una

via che dipende dall'attivazione di Ras (Califano *et al.* 1996; Califano *et al.* 2000). Allo scopo di investigare il ruolo di Shp-2 nel segnale intracellulare mediato dal recettore RET, abbiamo determinato l'induzione dell'espressione dei geni *Egr-1* e *c-fos*, mediata dai mutanti di RET, in presenza di un dominante negativo della fosfatasi Shp-2. Questo dominante negativo, Shp-2(c/s), inibisce l'attività biologica di Shp-2 perché presenta una mutazione puntiforme, che consiste nella sostituzione di un residuo di cisteina con un residuo di serina in posizione 459, nel dominio catalitico. Il mutante Shp-2(c/s), sebbene sia cataliticamente inattivo, è in grado di legare substrati proteici. Per i nostri studi abbiamo usato i plasmidi *Egr-1*-CAT e *c-fos*-CAT nei quali l'espressione di un "reporter" proteico, la proteina CAT (cloramfenicolo-acetil-transferasi), è sotto il controllo del promotore del gene *Egr-1* oppure del gene *c-fos*. Cellule PC12 sono state trasfettate transientemente con il plasmide *Egr-1*-CAT in presenza del vettore d'espressione per i mutanti del recettore RET (RET^{C634Y} oppure RET^{M918T}) e quantità crescenti del vettore di espressione per il dominante negativo Shp-2(c/s). Come è mostrato nella figura 21, il dominante negativo di Shp-2(c/s) inibisce l'attività della proteina reporter CAT mediata dai mutanti della proteina RET, sia quando la sua espressione è sotto il controllo del promotore del gene *Egr-1* (A, pannelli di sinistra e centrale) che quando la sua espressione è sotto il controllo del promotore del gene *c-fos* (B, pannelli di sinistra e centrale). L'inibizione arriva fino al

75% per *Egr-1* e più del 95% per *c-fos*. Come controllo positivo, la stimolazione delle cellule PC12 con il fattore di crescita NGF attiva l'espressione della proteina reporter CAT e questa attivazione diminuisce in presenza del dominante negativo Shp-2(c/s) (A e B, pannelli di destra). La trasfezione in presenza della proteina Shp-2 wt non ha indotto nessun cambiamento nei livelli di induzione della proteina "reporter" (dato non mostrato).

L'attività di Shp-2 è quindi necessaria, nel nostro sistema, all'induzione dei promotori di *Egr-1* e *c-fos*. Questi risultati indicano che la fosfatasi Shp-2 partecipa come effettore positivo nell'attivazione della via Ras/MAP chinasi indotta sia dal mutante RET^{C634Y} che RET^{M918T}.

8. La fosfatasi Shp-2 media la sopravvivenza cellulare indotta dal mutante RET^{M918T}

È noto che l'attivazione della via PI3K/AKT è fondamentale per la capacità trasformante della proteina RET (Segouffin-Cariou *et al.* 2000). Ci siamo chiesti se l'interazione di Shp-2 con il recettore RET potesse mediare l'induzione della cascata PI3K/AKT. Usando tecniche che identificano la frammentazione del DNA abbiamo dimostrato che in cellule PC12/wt, PC12/Ret^{C634Y} e PC12/Ret^{M918T}, l'espressione di RET^{C634Y} e di RET^{M918T} protegge le cellule PC12 dall'apoptosi indotta dall'eliminazione di siero dal

mezzo di crescita (figura 22, A e B). Inoltre, come è mostrato in figura, la protezione dell'apoptosi mediata dal mutante RET^{M918T} è più forte rispetto alla protezione dell'apoptosi mediata dal mutante RET^{C634Y}. Poiché durante la protezione dall'apoptosi è attivata la via di PI3K/AKT (De Vita *et al.* 2000), ci siamo chiesti se l'attività biologica della fosfatasi Shp-2 potesse essere coinvolta nell'attivazione di questa via. A tale scopo, sono state ottenute linee cellulari PC12/RetC634Y^{Shp-2(cs)} e PC12/RetM918T^{Shp-2(cs)}, trasfettando stabilmente le cellule PC12/Ret^{C634Y} e PC12/Ret^{M918T} con il vettore d'espressione per la proteina Shp-2(c/s). I lisati cellulari di cellule PC12-α1/wt, PC12/Ret^{C634Y}, PC12/Ret^{M918T}, PC12/RetC634Y^{Shp-2(cs)} e PC12/RetM918T^{Shp-2(cs)} sono stati analizzati per i livelli di fosforilazione della serina 473 di AKT, come una indicazione della sua attivazione (Datta *et al.* 1999). Come è mostrato nella figura 23-A, in cellule PC12-α1/wt stimulate con GDNF, in cellule PC12/Ret^{C634Y} e in cellule PC12/Ret^{M918T}, aumentano i livelli di fosforilazione di Akt sulla serina 473. Inoltre, l'espressione stabile di Shp-2(c/s) nelle cellule PC12/RetC634Y^{Shp-2cs} e PC12/RetM918T^{Shp-2cs} riduce notevolmente l'attivazione di AKT. Per evitare possibili artefatti causati dalla selezione di cloni stabili ottenuti trasfettando RET^{C634Y}, RET^{M918T} e Shp-2(c/s) nelle linee cellulari parentali, abbiamo valutato gli effetti di Shp-2 sull'attività di AKT con esperimenti di trasfezione transiente nella linea cellulare parentale PC12. Cellule PC12 sono state trasfettate con un vettore d'espressione per la proteina AKT-HA

in presenza del vettore d'espressione per i mutanti di RET (RET^{C634Y} che RET^{M918T}), e gli estratti cellulari sono stati analizzati per la presenza della proteina AKT fosforilata sulla serina 473. Come è mostrato in B, la fosforilazione di AKT è stimolata dalla presenza del mutante RET^{M918T}, ma rimane bassa dopo l'espressione del mutante RET^{C634Y}. Questo risultato indica che, in queste condizioni sperimentali, la via di trasduzione del segnale di AKT è attivata dal mutante RET^{M918T}. A questo punto, per studiare il coinvolgimento di Shp-2 nell'attivazione di AKT mediata da RET^{M918T}, abbiamo trasfettato le cellule PC12 con i vettori d'espressione AKT-HA e RET^{M918T} in presenza del dominante negativo della fosfatasi, Shp-2(c/s). Come è mostrato in figura, l'espressione di Shp-2(c/s) riduce notevolmente sia la fosforilazione (C) che l'attività catalitica (D) della chinasi AKT.

La proteina Bad è un membro, con attività pro-apoptotica, della famiglia Bcl-2. Quando AKT è attivato dalla fosforilazione della serina 473, fosforila Bad inibendone la sua attività apoptotica (Datta *et al.* 1999). Abbiamo, quindi, controllato i livelli di fosforilazione della proteina Bad trasfettando transientemente le cellule PC12 con il vettore d'espressione per la proteina in presenza del vettore d'espressione per i mutanti di RET (RET^{C634Y} e RET^{M918T}). Abbiamo determinato i livelli di fosforilazione di Bad per immunoblot con anticorpi anti fosfo-Bad. Come ci aspettavamo, l'espressione del recettore RET^{M918T} induce la fosforilazione di Bad sulle

serine 112 e 136 (E), e questa fosforilazione è ridotta dalla presenza della fosfatasi Shp-2(c/s) come dominante negativo (F). Questi risultati indicano, quindi, che in cellule PC12 la fosfatasi Shp-2 è coinvolta nell'attivazione della via di trasduzione del segnale PI3K/AKT mediata dal recettore RET^{M918T}.

9. La fosfatasi Shp-2 media il segnale indotto dal GDNF

Allo scopo di confermare il coinvolgimento della fosfatasi Shp-2 nella trasduzione del segnale mediata dal recettore RET, abbiamo stimolato le cellule PC12- α 1/wt, che esprimono l'allele normale del recettore RET e il co-recettore GFR α -1, con il fattore di crescita GDNF. In queste cellule, dopo la stimolazione con GDNF, RET induce sia l'espressione dei "immediate early" geni *Egr-1* e *c-fos* coinvolti nella differenziazione cellulare, che la fosforilazione della chinasi AKT. Come è mostrato nei dosaggi CAT in figura 24 A, in cellule PC12- α 1/wt stimulate con GDNF, sia l'induzione del promotore del gene *Egr-1* che del gene *c-fos* è inibita dall'espressione del dominante negativo Shp-2(cs). Inoltre, abbiamo trasfettato transientemente, nella stessa linea cellulare, il vettore d'espressione per la proteina Akt-HA, ed abbiamo analizzato i livelli di induzione della fosforilazione di Akt sulla serina 473 da parte del GDNF. Come è mostrato in B la stimolazione di RET induce un aumento della fosforilazione di Akt di circa tre volte. Nel caso di RET^{wt} l'attivazione di

RET non sembra mediata dall'attività di Shp-2, infatti l'espressione del dominante negativo Shp-2(c/s) non ha effetto sulla fosforilazione di Akt. Questi risultati indicano che Shp-2 media la via dipendente da Erk per l'attivazione di *Egr-1* e *c-fos*, sia dopo uno stimolo acuto (in presenza di GDNF), che dopo l'induzione mediata dai mutanti costitutivi di RET (RET^{C634Y} e RET^{M918T}), mentre può mediare l'attivazione della via di AKT solo in presenza di uno stimolo cronico indotto dai mutanti di RET.

DISCUSSIONE

Shp-2 è una fosfatasi espressa ubiquitariamente e la sua attività biologica è richiesta per mediare la trasmissione di segnali intracellulari innescati dall'attivazione di diversi recettori ad attività tirosina chinasi. La fosfatasi Shp-1, espressa preferenzialmente in cellule di derivazione ematopoietica ed epiteliale, si comporta come un effettore negativo nei segnali intracellulari innescati da differenti recettori di membrana.

Mutazioni del gene *ret*, che inducono l'attivazione costitutiva del recettore in assenza di stimolazione, sono responsabili di neoplasie ereditabili come la MEN2A, MEN2B e il FMTC. La MEN2A è determinata da mutazioni puntiformi nel dominio extracellulare ricco in cisteine del recettore (es. C634Y). Nella MEN2B le mutazioni ricadono nel dominio intracellulare di RET (es. M918T).

Per i nostri studi abbiamo utilizzato, come sistema modello, cellule PC12/Ret^{C634Y} e PC12/Ret^{M918T} che esprimono le fosfatasi Shp-1 e Shp-2. Le due linee cellulari sono state ottenute trasfettando stabilmente la linea parentale PC12 con i vettori d'espressione per i mutanti di RET, rispettivamente Ret^{C634Y} e Ret^{M918T}.

Dopo stimolazione da GDNF il recettore RET trasloca nei domini di membrana "lipid rafts". La localizzazione di Ret in questi domini, gli conferisce la possibilità di attivare dei substrati proteici che sono differenti rispetto a quelli localizzati e reclutati esternamente ai "lipid rafts". Sono

stati formulati due meccanismi circa la localizzazione di Ret attivato nei lipid rafts: il primo implica l'interazione fisiologica tra la regione extracellulare di Ret e il GFR α -1, il secondo richiede l'interazione tra il dominio intracellulare tirosino chinasi di Ret con i substrati localizzati nei lipid rafts (Paratcha *et al.* 2001).

In accordo con i dati appena citati, nei nostri studi abbiamo dimostrato che anche i mutanti di RET, Ret^{C634Y} e Ret^{M918T}, sono localizzati sia nei "lipid rafts" che nelle frazioni del gradiente di saccarosio che escludono i "lipid rafts". Inoltre, abbiamo dimostrato che esiste una diversa distribuzione di membrana delle molecole Ret^{C634Y} e Ret^{M918T} fosforilate in tirosina. Il recettore Ret^{C634Y} fosforilato in tirosina è localizzato esclusivamente nei "lipid rafts" mentre il recettore Ret^{M918T} fosforilato in tirosina è distribuito equamente nelle diverse frazioni del gradiente di saccarosio.

Poiché i mutanti di RET sono attivi in assenza di stimolazione con GDNF, abbiamo ipotizzato che la localizzazione di membrana del recettore, potesse essere mediata dalla sua interazione con distinti substrati proteici presenti in questi domini.

Gli studi condotti nel nostro laboratorio hanno dimostrato che, sia in cellule PC12/Ret^{C634Y} che in cellule PC12/Ret^{M918T}, le tirosino fosfatasi Shp-1 e Shp-2 interagiscono con i mutanti attivi di RET formando complessi molecolari distinti che contengono Shp-1 o Shp-2 ma non le due fosfatasi insieme. Inoltre, questi complessi localizzano in compartimenti distinti

della membrana plasmatica. Infatti, Shp-1 può legare RET mutato sia dentro che fuori i “lipid rafts” anche se il legame è favorito nei domini di membrana che includono i “lipid rafts”. Viceversa, la fosfatasi Shp-2 lega RET mutato solo ed esclusivamente in domini di membrana che escludono i “lipid rafts”. Come è riportato in letteratura, le due fosfatasi si differenziano nella funzione biologica in quanto Shp-1 funziona come effettore negativo mentre Shp-2 come effettore positivo in diverse vie di trasduzione del segnale. Si potrebbe ipotizzare che anche nel segnale mediato da RET, per effetto della loro differente localizzazione di membrana, Shp-1 e Shp-2 possano comportarsi come dirette antagoniste l’una dell’altra. Questa ipotesi può essere confermata sia dai risultati ottenuti da altri gruppi di ricerca che dai risultati ottenuti nel nostro laboratorio. Infatti, Hannige e colleghi hanno dimostrato che in cellule NIH3T3, stabilmente trasfettate con un mutante attivo di RET (Ret9bp), Shp-1 ma non Shp-2, partecipa come effettore negativo nella differenziazione e proliferazione cellulare mediata da RET (Hennige *et al.* 2001). D’altra parte, i nostri risultati hanno dimostrato che, in cellule PC12, l’attività biologica di Shp-2 è richiesta per la differenziazione e la proliferazione cellulare mediata dai mutanti di RET. Se la nostra ipotesi è corretta, l’arricchimento nei lipid rafts di molecole di Shp-1 legate ad entrambi i mutanti di RET potrebbe rappresentare parte del suo meccanismo negativo. La definizione dei reali substrati delle due fosfatasi

può darci la possibilità di chiarire il motivo per cui Shp-1 e Shp-2 localizzano in diversi domini della membrana plasmatica.

Nell'insieme, questi risultati indicano che Shp-1 e Shp-2 legano RET in complessi multiproteici localizzati in differenti compartimenti di membrana e che le due fosfatasi non fanno parte dello stesso complesso multiproteico. Questa differente localizzazione di membrana dei complessi si associa a due distinte funzioni biologiche: per Shp-2 quella di rispondere positivamente all'attività tirosina chinasi del recettore Ret mentre per Shp-1 quella di inibire, attraverso un meccanismo ancora sconosciuto, i segnali mediati dal recettore RET

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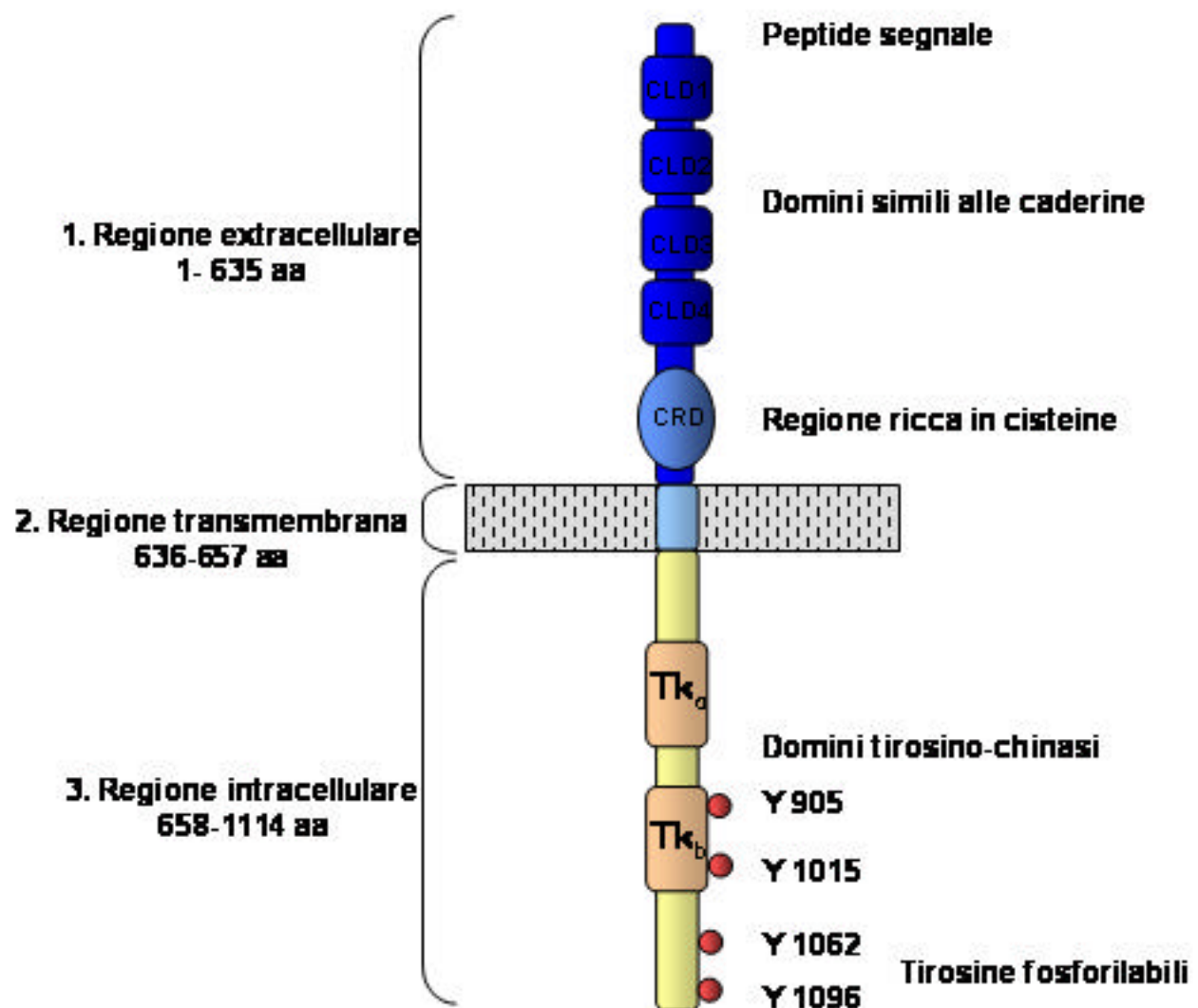


Fig. 1 La struttura del recettore tirosino-chinasi RET

RET è un recettore transmembrana organizzato in tre regioni 1) extracellulare; 2) transmembrana; 3) intracellulare. La regione extracellulare comprende 635 aminoacidi, e possiede: un peptide segnale che dirige la proteina alla membrana; quattro domini simili alle caderine; una regione ricca in residui di cisteina. La regione transmembrana è composta da 21 aminoacidi. La regione intracellulare può essere di varie dimensioni a seconda dell'isoforma di Ret, RET51 comprende 456 aminoacidi. In questa porzione sono presenti due domini catalitici tirosino-chinasici e le tirosine fosforilabili.

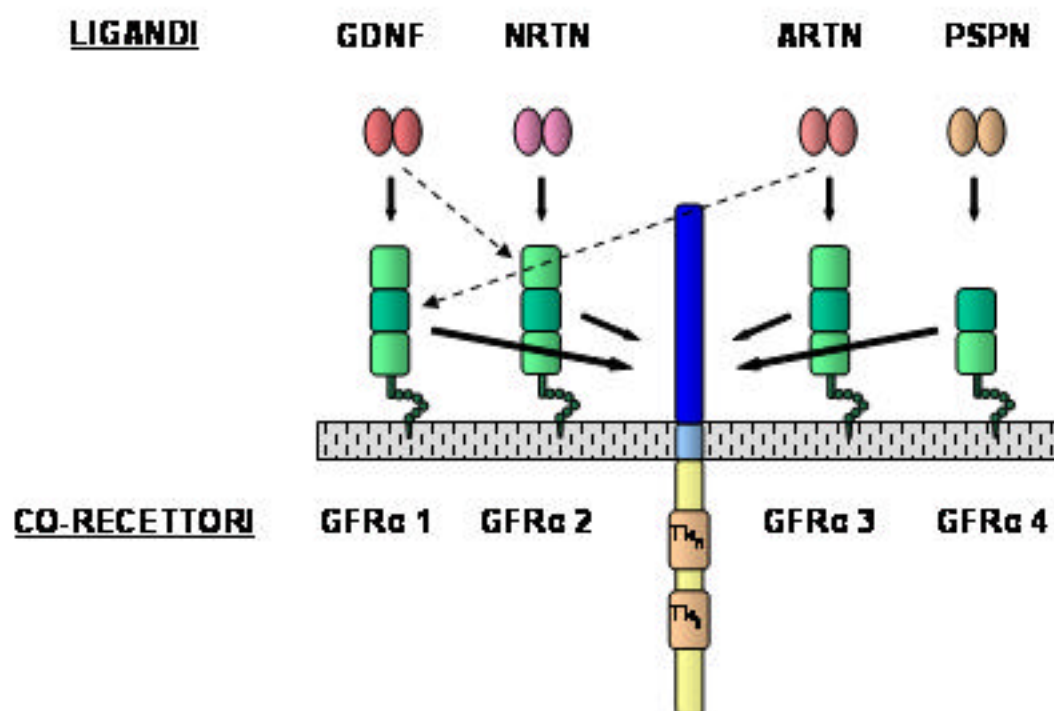


Fig. 2 I ligandi e i co-recettori si associano a RET durante l'attivazione del recettore

I ligandi della famiglia del GDNF legano i co-recettori, membri della famiglia del GFR α , in maniera selettiva ma non totalmente esclusiva.

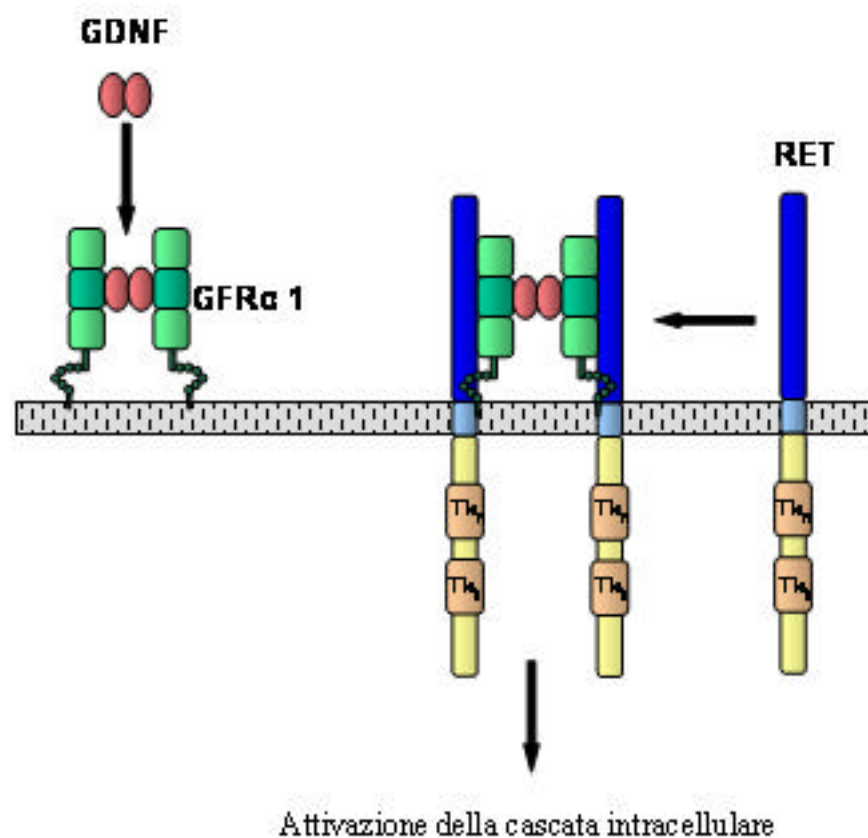


Fig. 3 Il complesso di trasduzione del segnale formato da Ret, il co-recettore GFRα-1 e il GDNF

La formazione del complesso GDNF/GFRα-1/RET media la trasduzione del segnale all'interno della cellula.

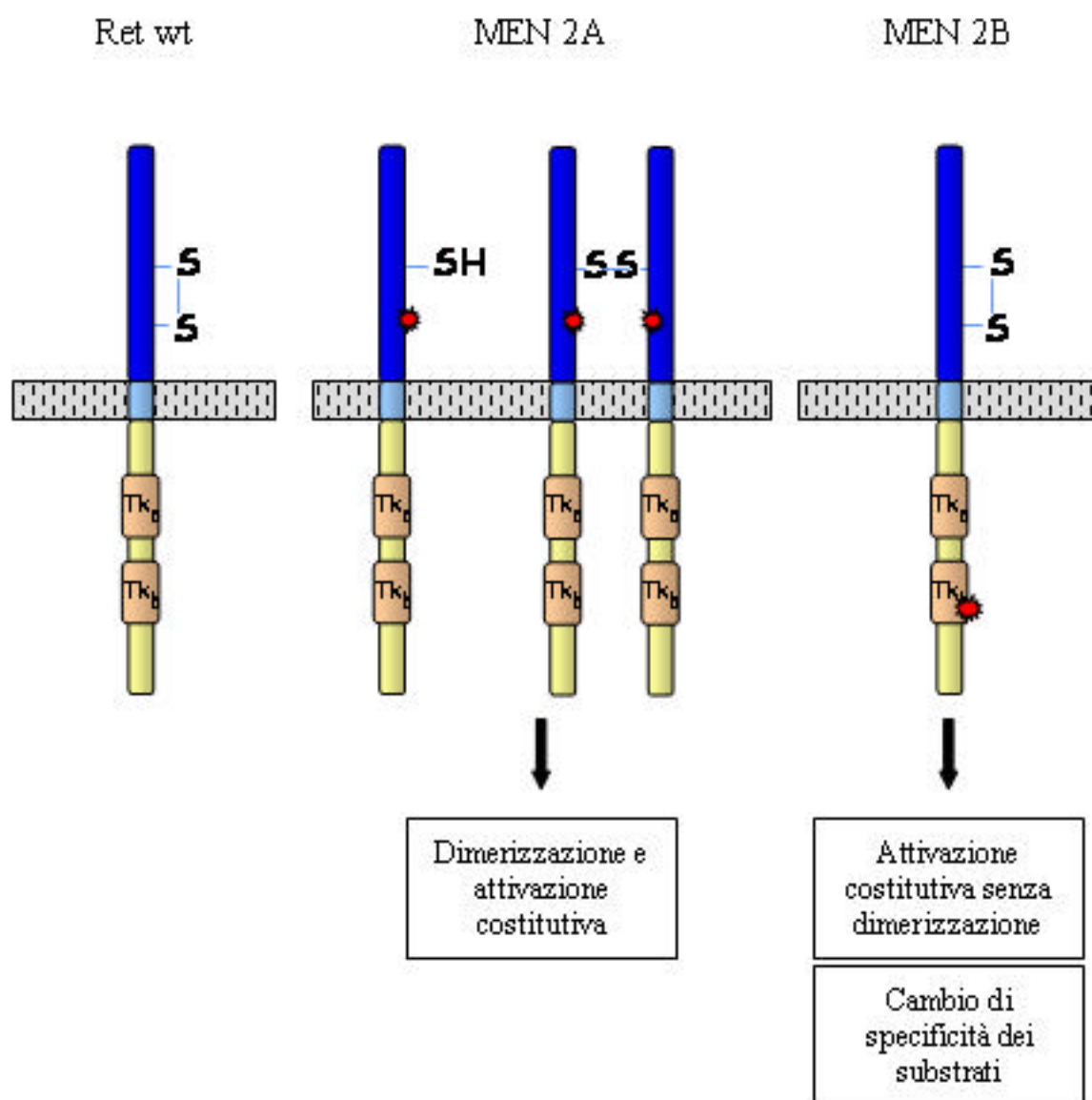


Fig. 4 Le mutazioni germinali di *ret* che causano le patologie MEN 2A e MEN 2B

MEN 2A è causata dalla sostituzione di un residuo di cisteine nella regione extracellulare del recettore. Questa sostituzione è responsabile della dimerizzazione, e quindi dell'attivazione costitutiva, del recettore in assenza del ligando. MEN 2B è causata da una mutazione nella regione intracellulare con conseguente attivazione costitutiva di Ret senza la dimerizzazione; inoltre la mutazione (quasi sempre la M918T) causa un cambiamento nella specificità del recettore per i substrati intracellulari.

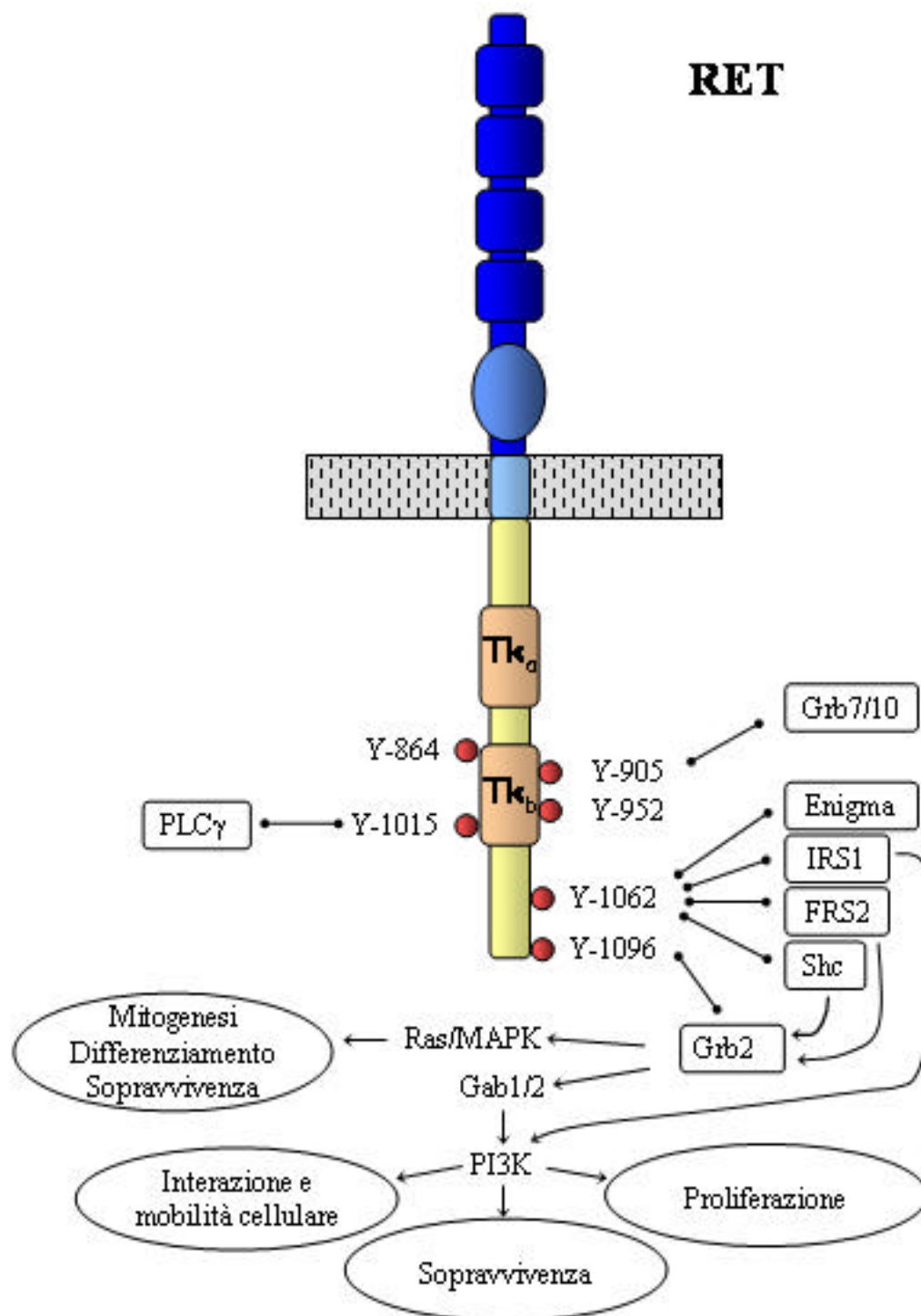


Fig. 5 Il segnale intracellulare innescato dall'attivazione del recettore Ret
 La formazione del complesso GDNF/GFR α -1/Ret provoca la dimerizzazione del recettore e l'autofosforilazione a livello delle tirosine localizzate nella regione intracellulare del recettore. Le tirosine fosforilate sono dei siti di reclutamento per le proteine adattatrici che collegano il recettore a varie vie di trasduzione intracellulare con differenti effetti sulla cellula. Sono mostrate solo le vie più conosciute.

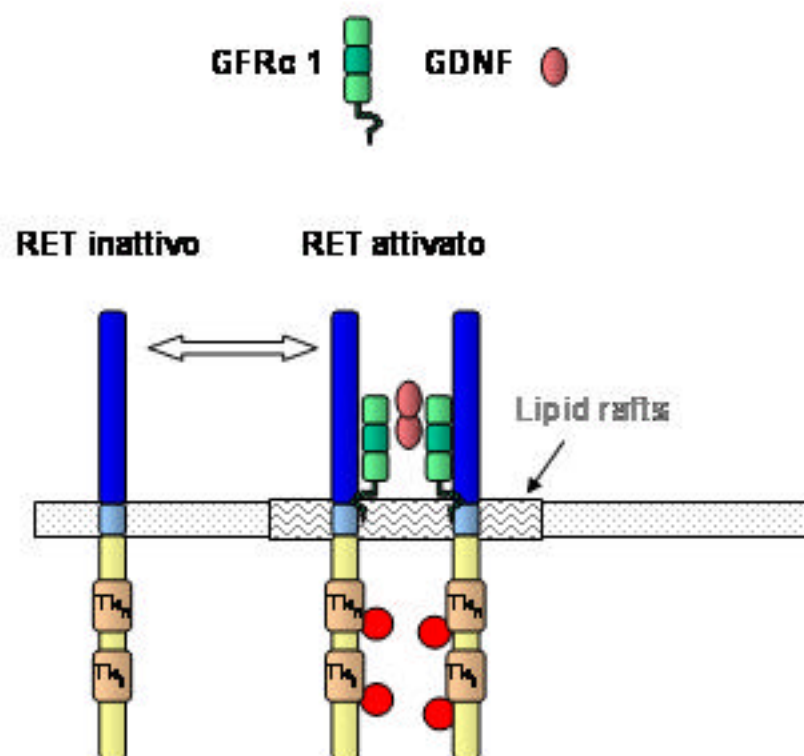


Fig. 6 La traslocazione del recettore RET nei "lipid rafts" in seguito alla stimolazione con il GDNF

Nella conformazione inattiva, il recettore RET è localizzato in domini di membrana che escludono i "lipid rafts". In seguito alla stimolazione di RET con GDNF il recettore trasloca nei "lipid rafts".

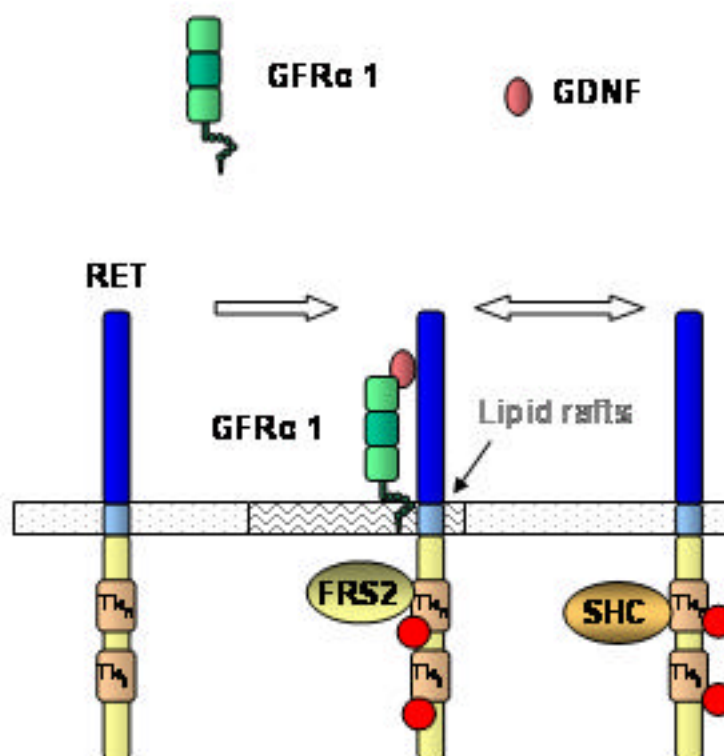


Fig. 7 Il recettore RET attivato, in seguito alla stimolazione con GDNF, localizza sia nei domini di membrana "lipid rafts" che in domini di membrana che escludono i "lipid rafts"

La localizzazione di RET in differenti domini di membrana media il riconoscimento del recettore con bersagli proteici distinti. RET attivato lega la proteina adattatrice FRS2 nei "lipid rafts" e la proteina adattatrice SHC in domini di membrana che escludono i "lipid rafts".

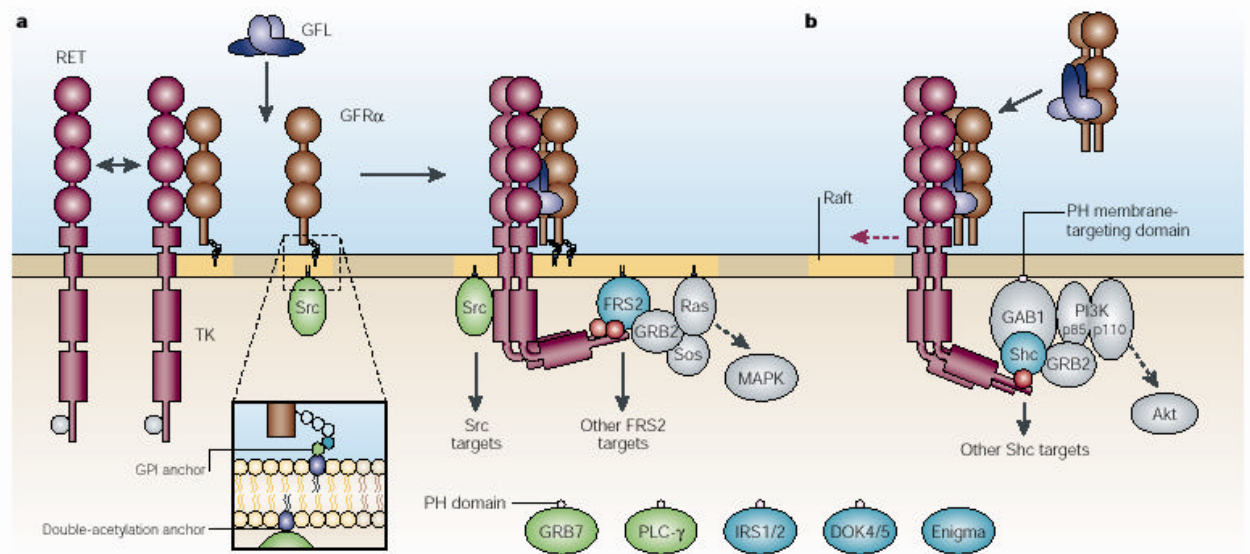
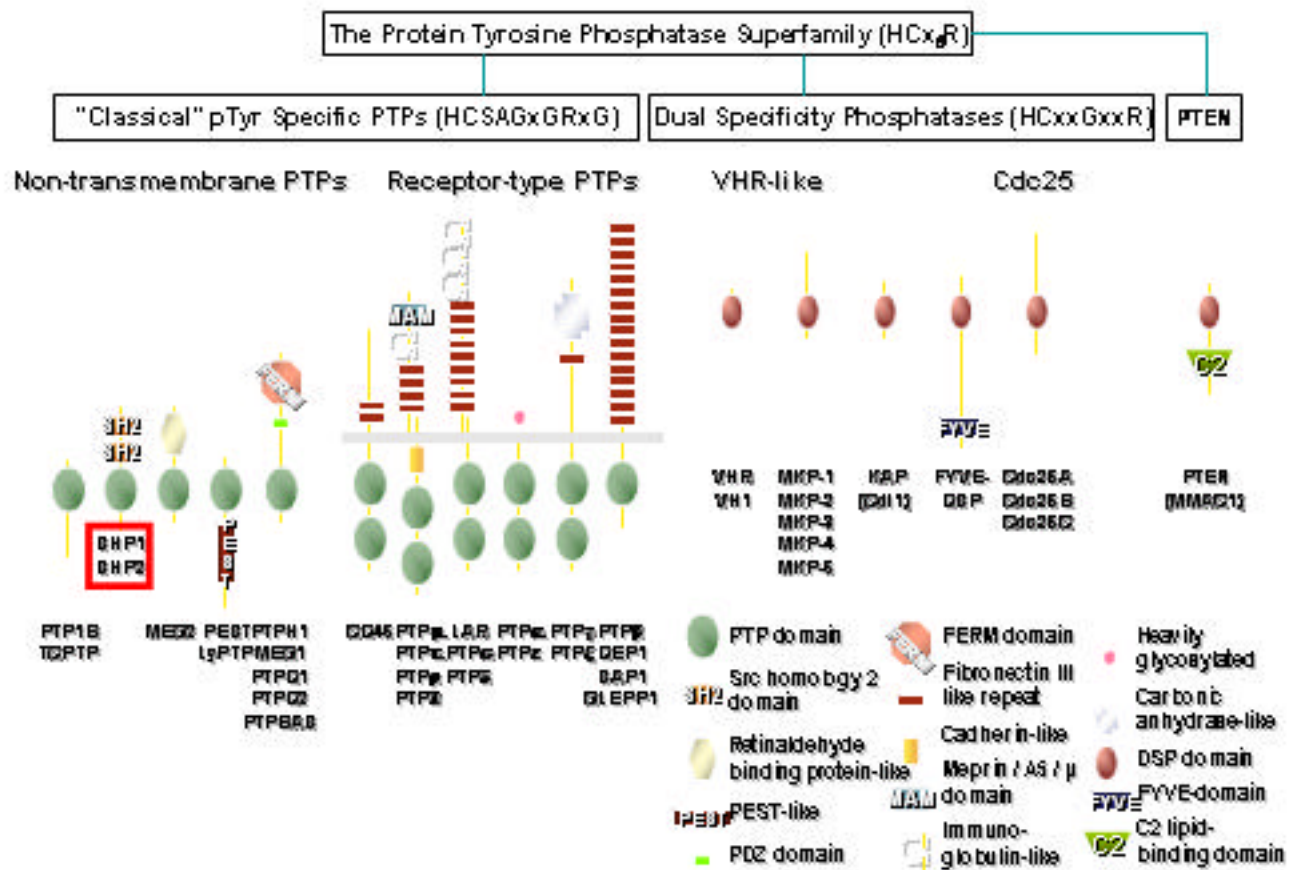


Fig. 8. I membri della famiglia del GDNF mediano distinti segnali dentro e fuori i “lipid rafts”. Il modello evidenzia le differenti proteine intracellulari che sono reclutate in membrana quando RET è stimolato nei lipid rafts e fuori i lipid rafts.



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Fig. 9 Rappresentazione schematica dei membri appartenenti alla superfamiglia delle tirosino fosfatasi

Le fosfatasi Shp-1 e Shp-2 sono evidenziate in rosso e sono classificate come tirosino Fosfatasi non-transmembrana

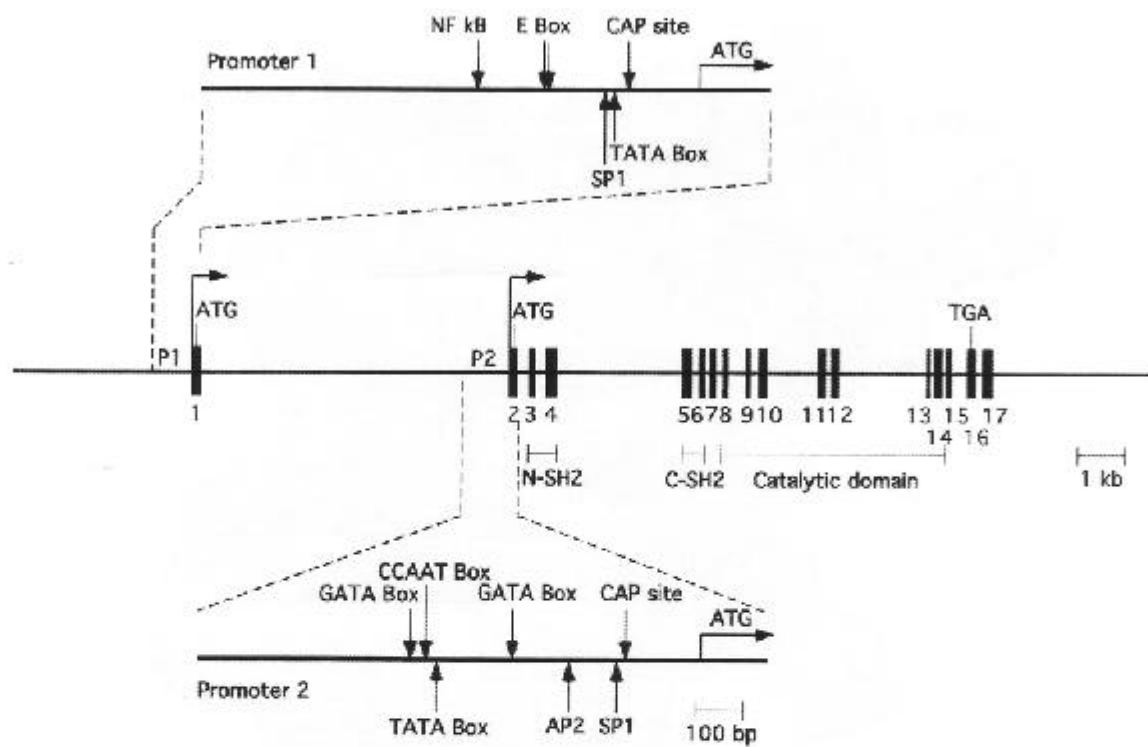


Fig. 10 Rappresentazione schematica del gene *Shp-1* umano

P1 e P2 indicano la localizzazione dei promotori 1 e 2 che sono attivi rispettivamente in cellule non-ematopoietiche e in cellule ematopoietiche. La rappresentazione schematica della regione dei promotori 1 e 2 è indicata nell'inserto.

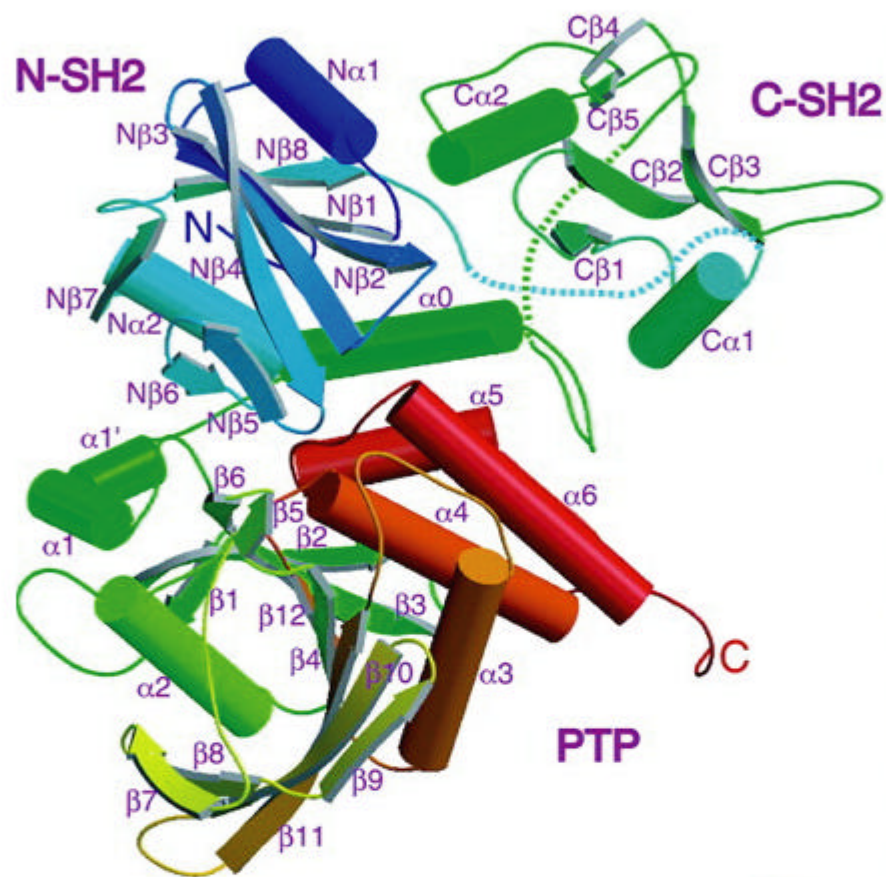


Fig. 11 Rappresentazione della struttura terziaria della proteina Shp-1
 La porzione in blu indica la regione N-terminale mentre la porzione in rosso indica la regione C-terminale. Nella regione all'N-terminale sono localizzati i domini N-SH2 e C-SH2.



Fig. 12 Rappresentazione della struttura terziaria della proteina Shp-2
 La regione in verde indica il dominio C-SH2, la regione in giallo indica il dominio N-SH2 e la regione in blu indica il dominio catalitico.

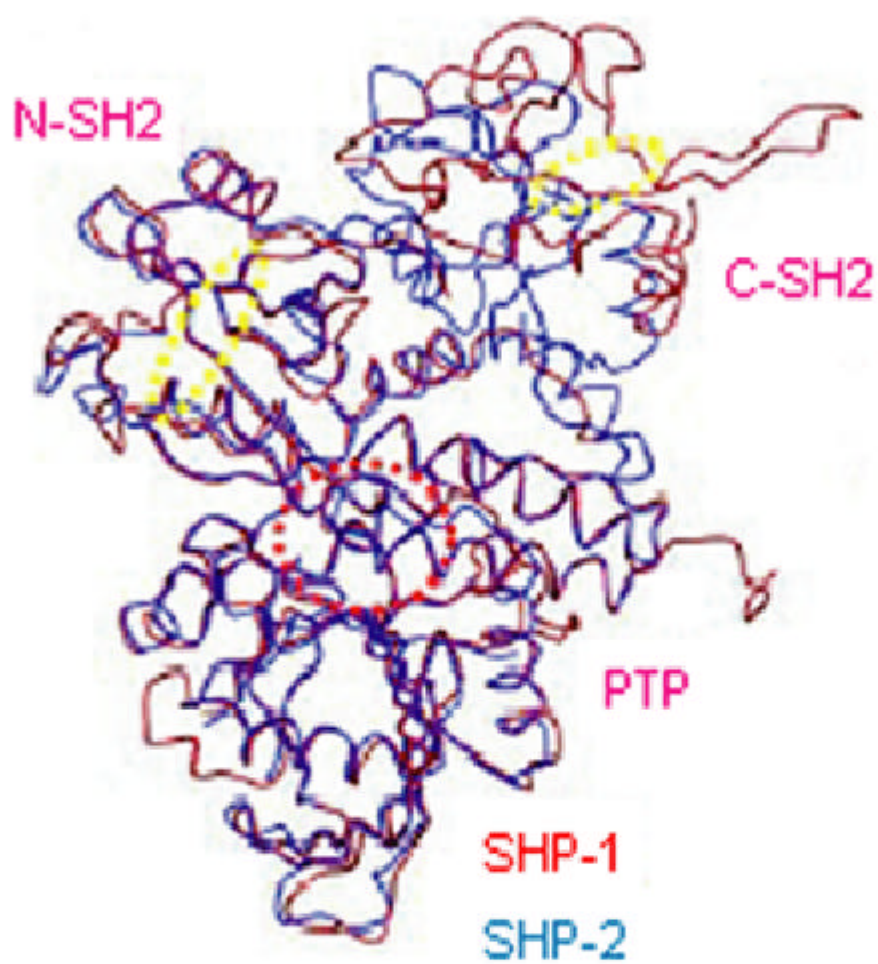


Fig. 13 Il confronto tra le strutture terziarie delle fosfatasi Shp-1 (blu) e Shp-2 (rosso)

Shps

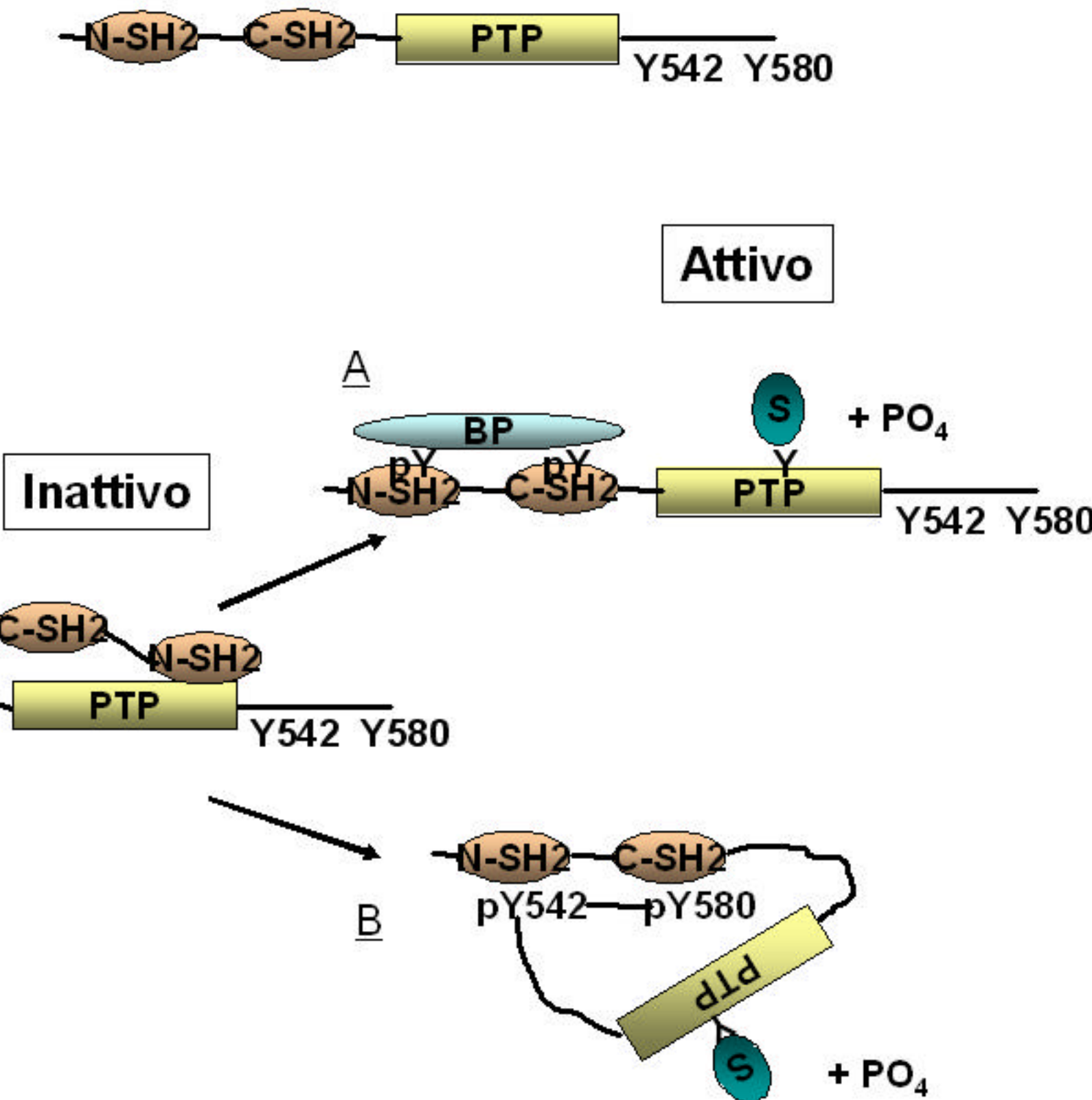


Fig. 14 Potenziale meccanismo di regolazione delle fosfatasi Shp-1 e Shp-2

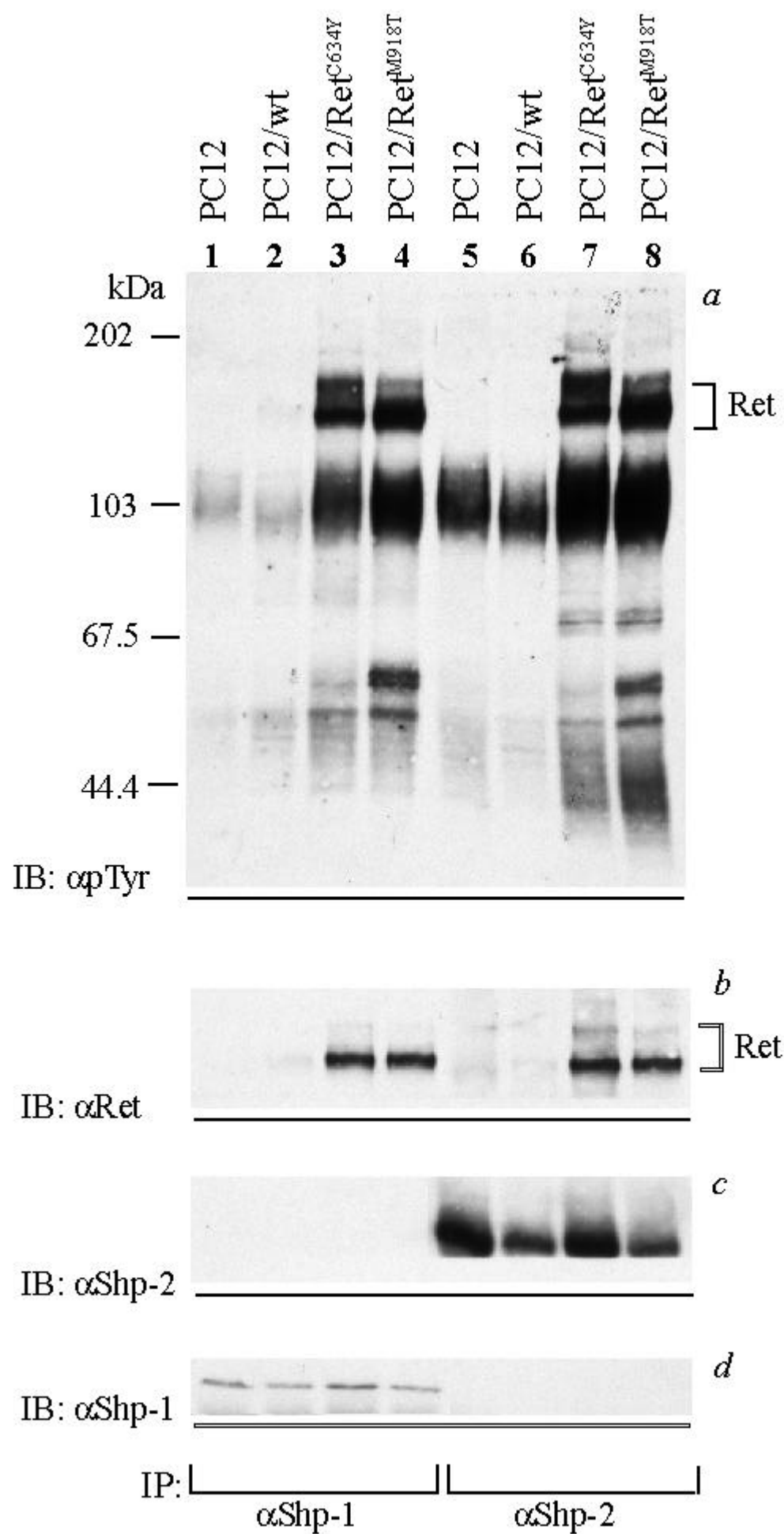
In assenza di stimolazione le fosfatasi sono in una conformazione inattiva poiché il dominio N-SH2 terminale interagisce con il dominio catalitico (PTP).

(A) attivazione delle fosfatasi mediante il legame dei due domini SH2 terminali alle tirosine fosforilate di una proteina bersaglio (BP)

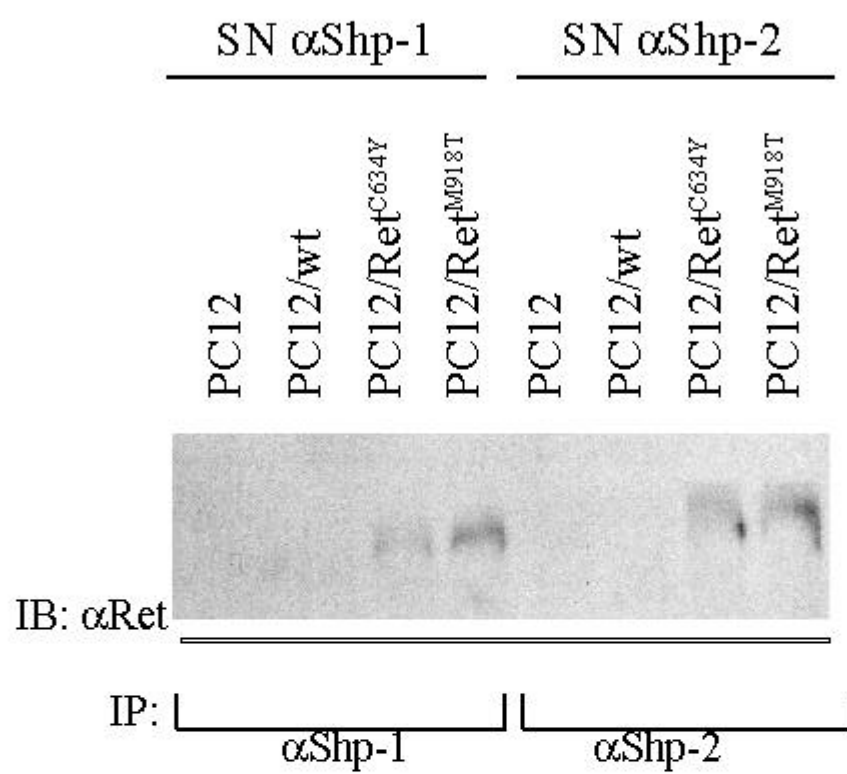
(B) attivazione intramolecolare delle fosfatasi mediante il legame delle tirosine fosforilate, in posizione 580 e 542, con i due domini SH2 terminali

In entrambi i meccanismi di attivazione il dominio catalitico è fisicamente liberato dall'interazione con il dominio N-SH2 terminale.

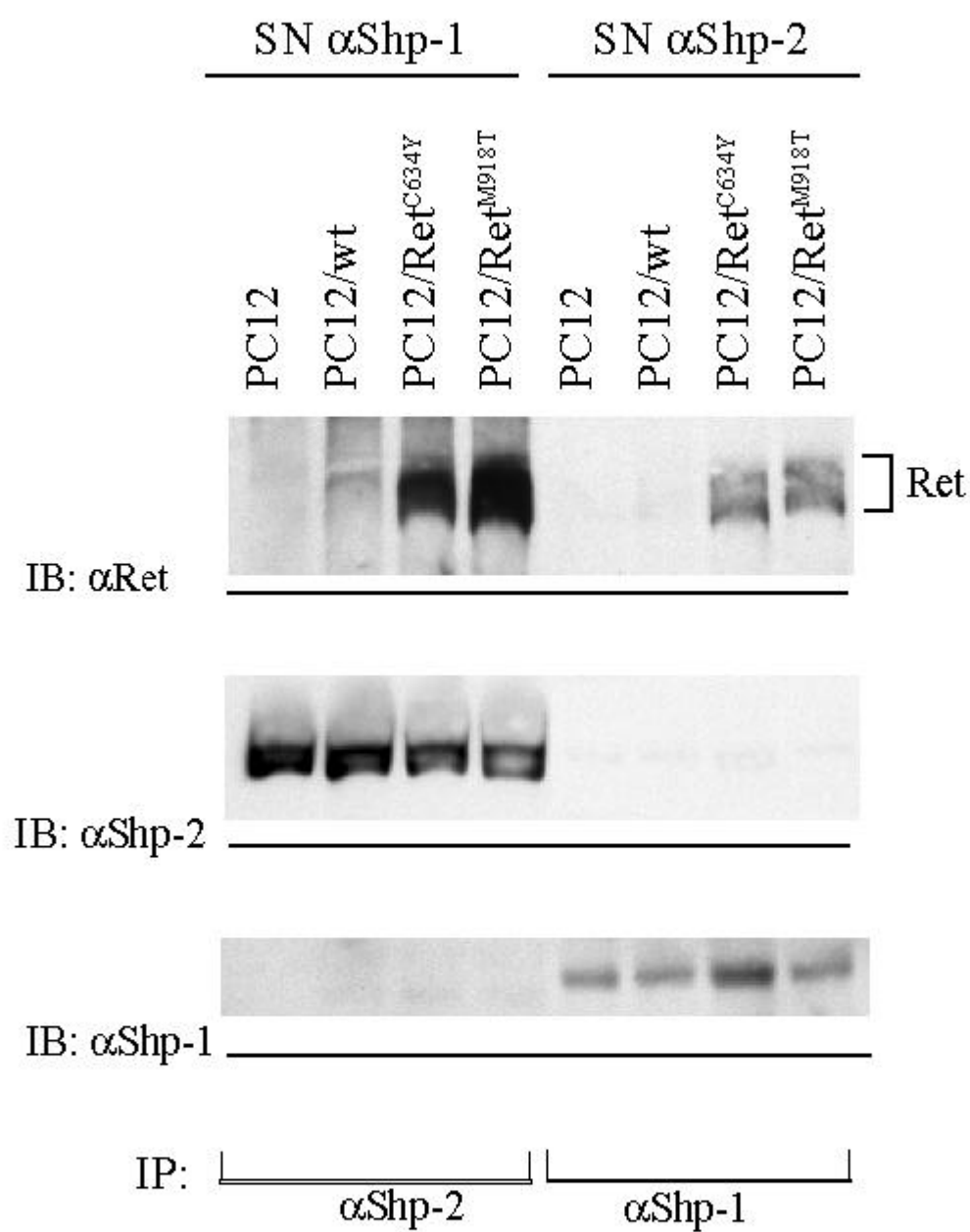
A

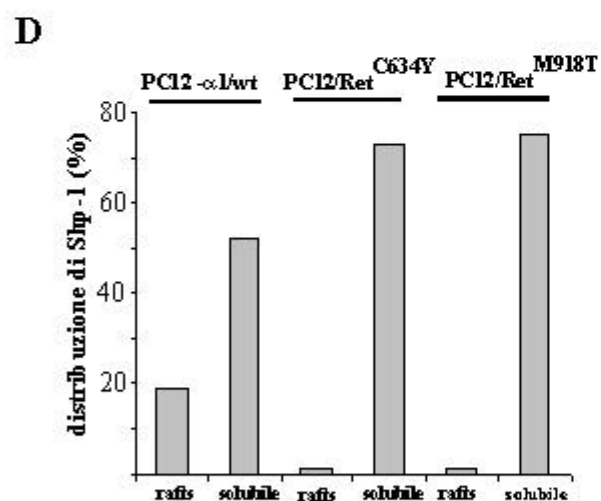
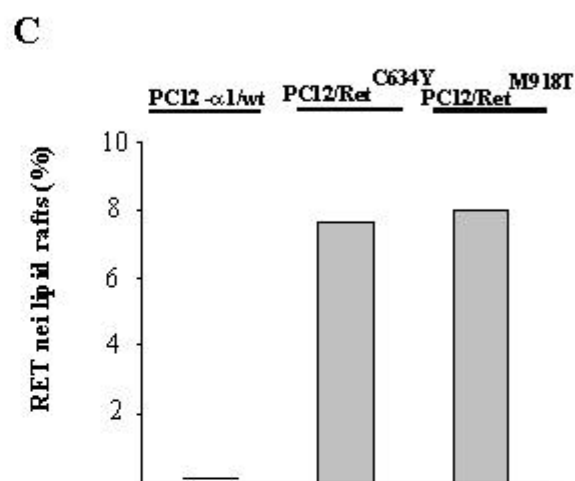
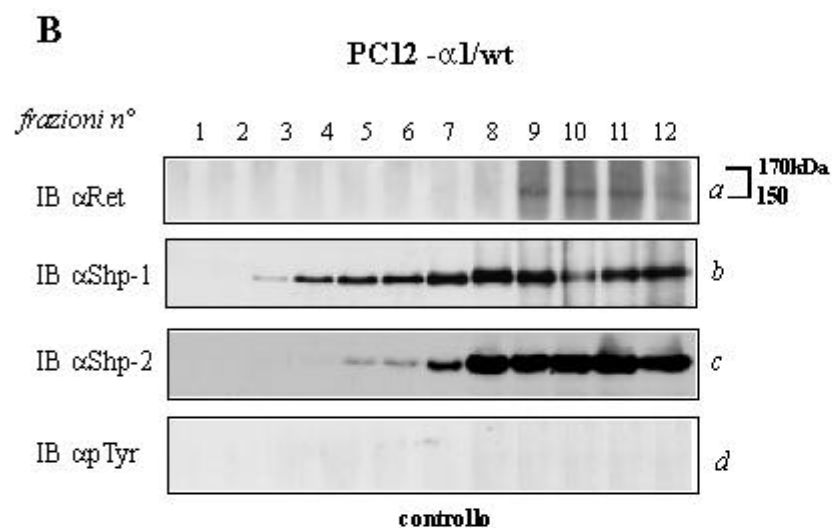
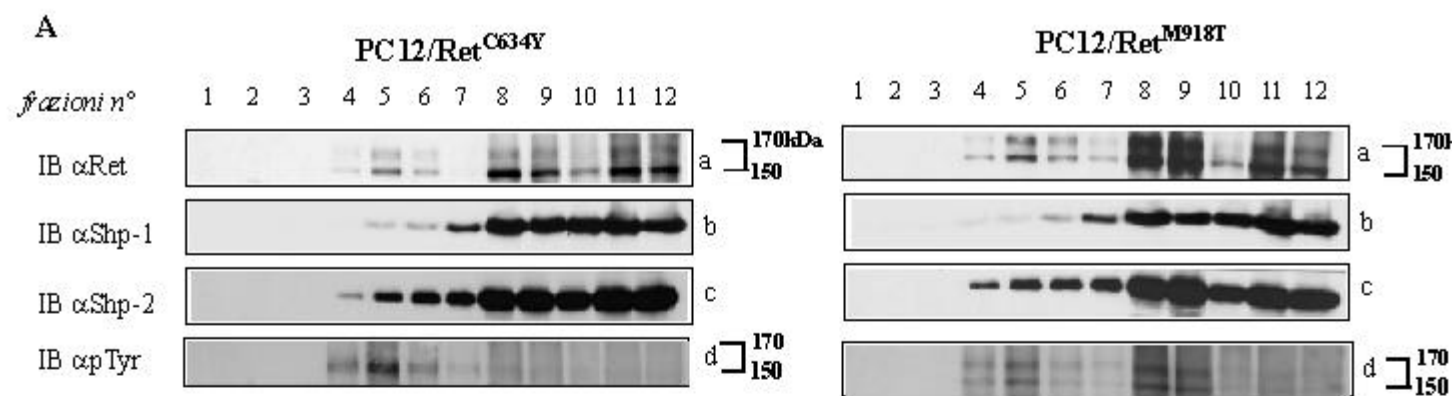


B



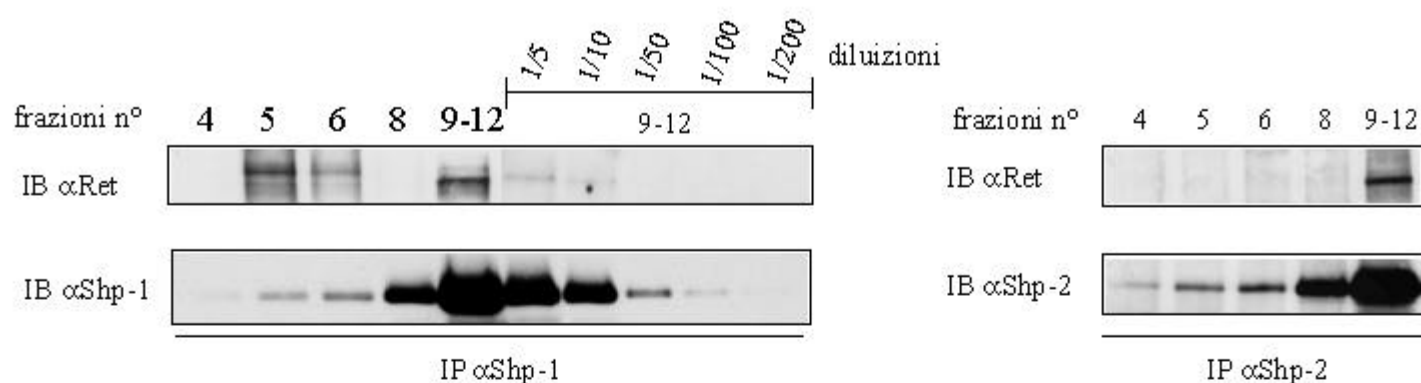
C





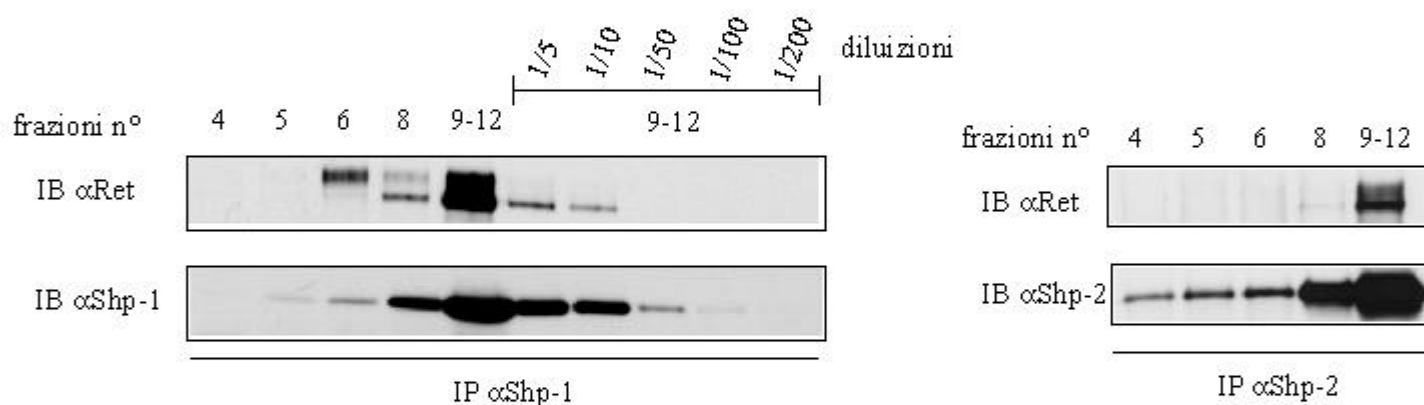
A

PC12/Ret^{C634Y}

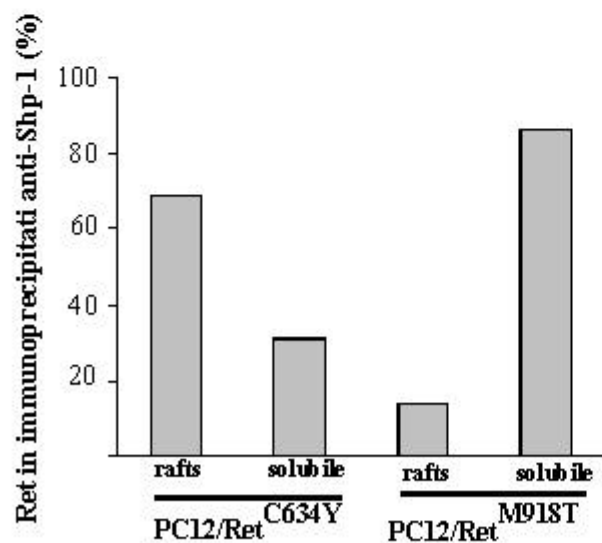


B

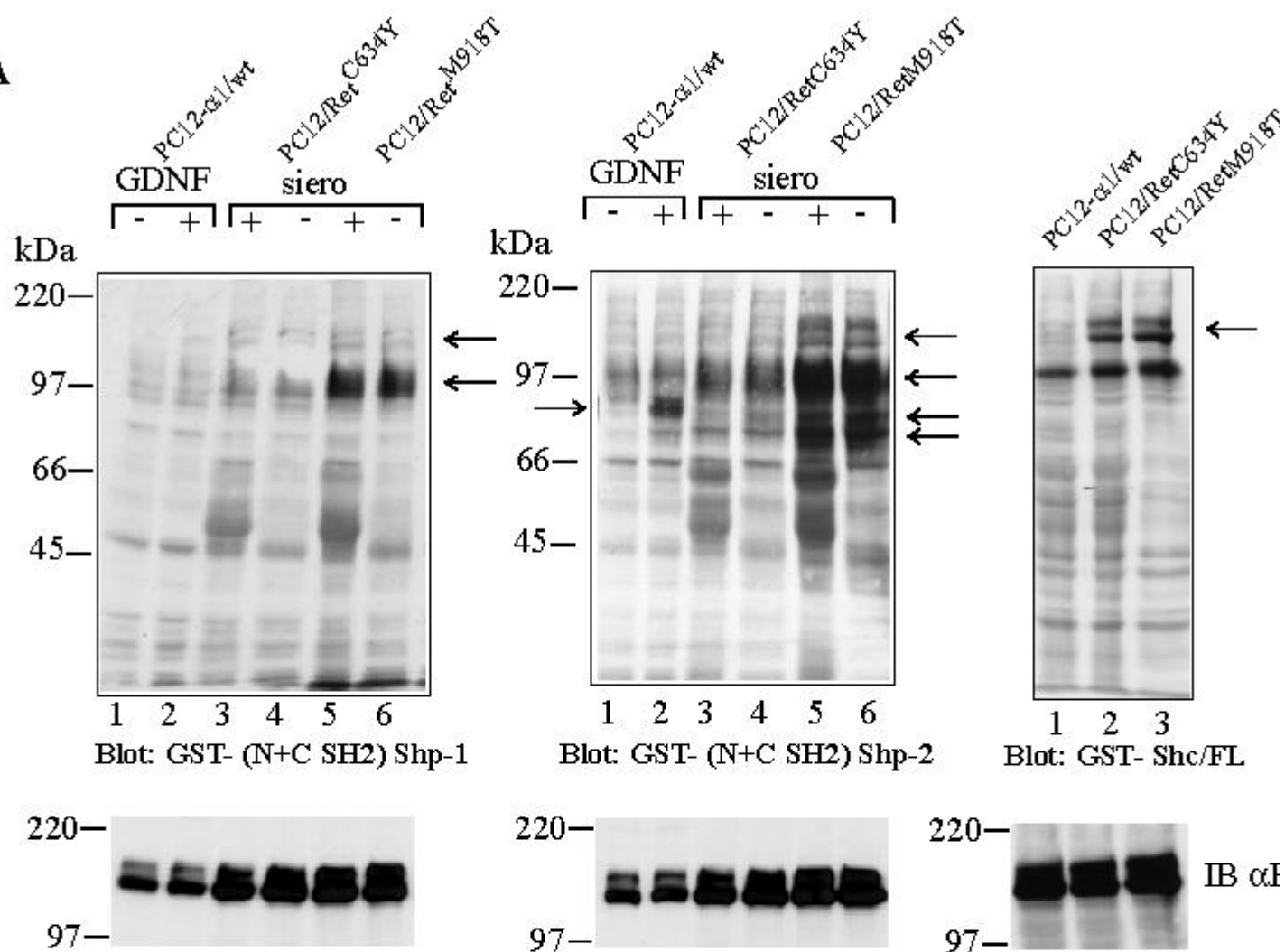
PC12/Ret^{M918T}



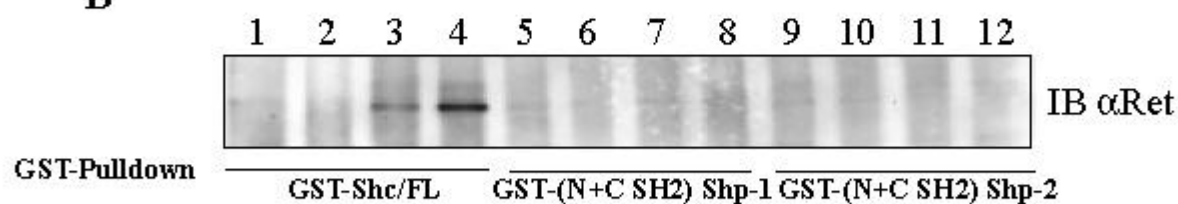
C



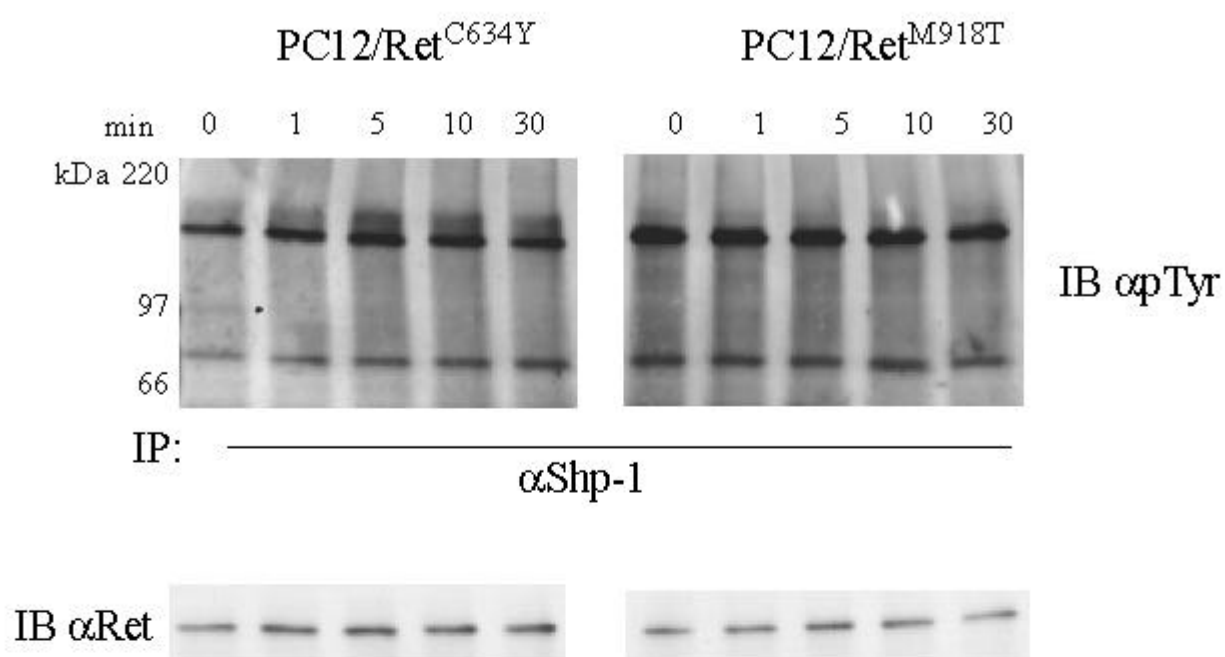
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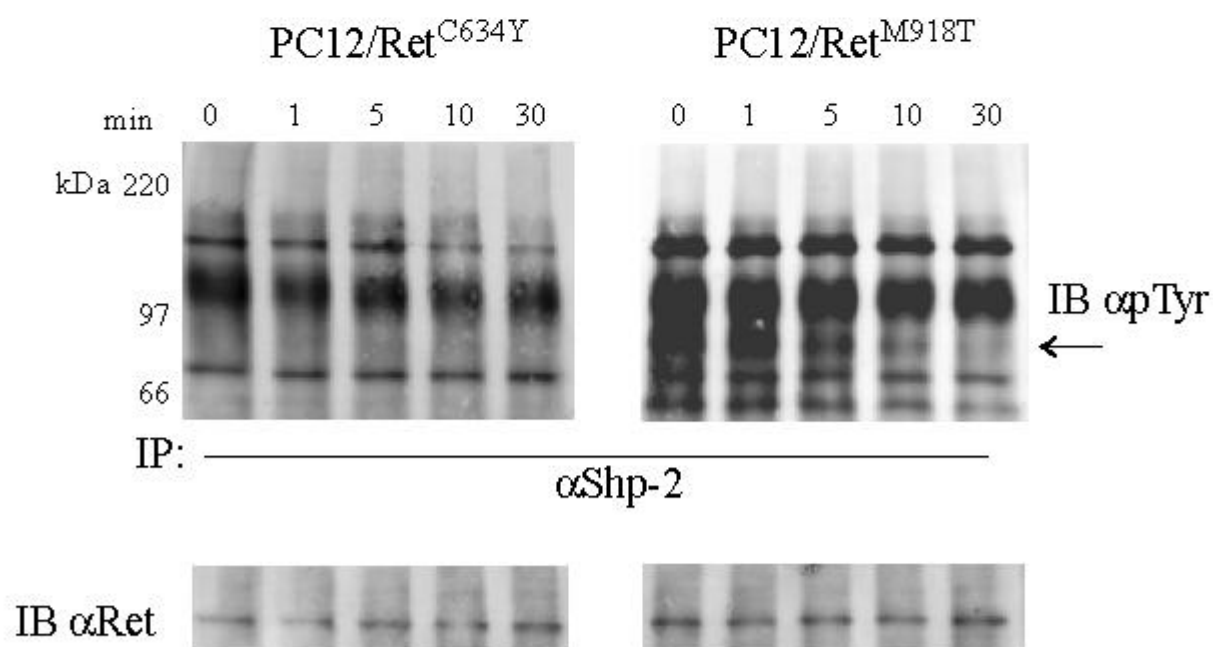
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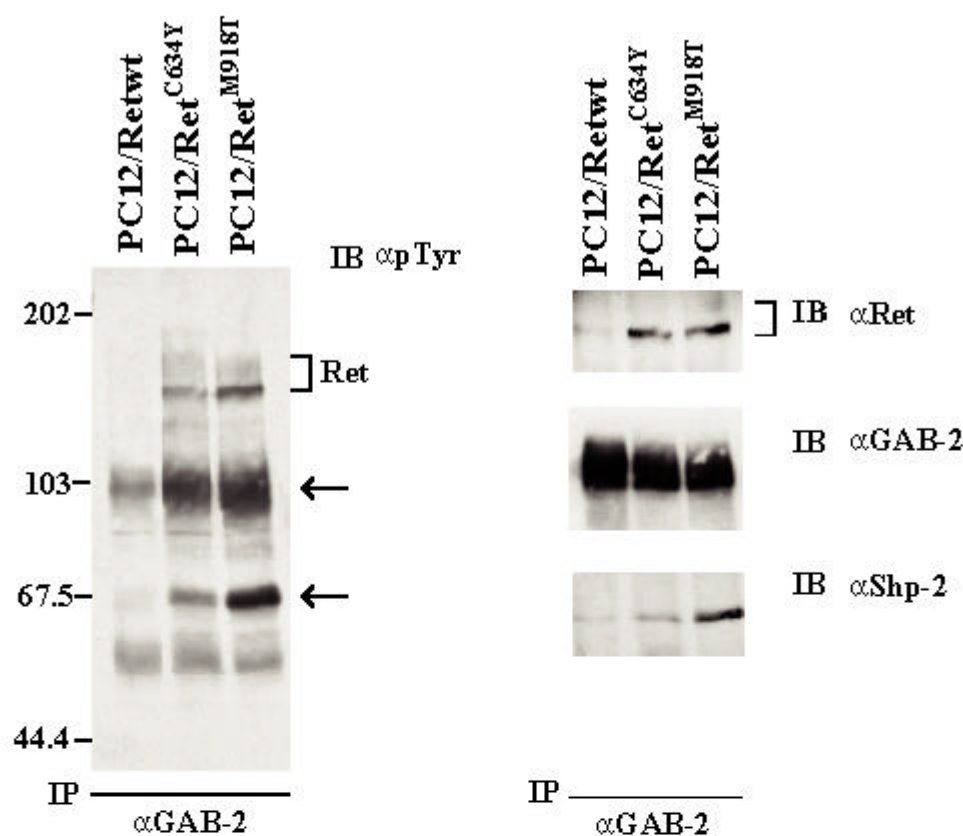
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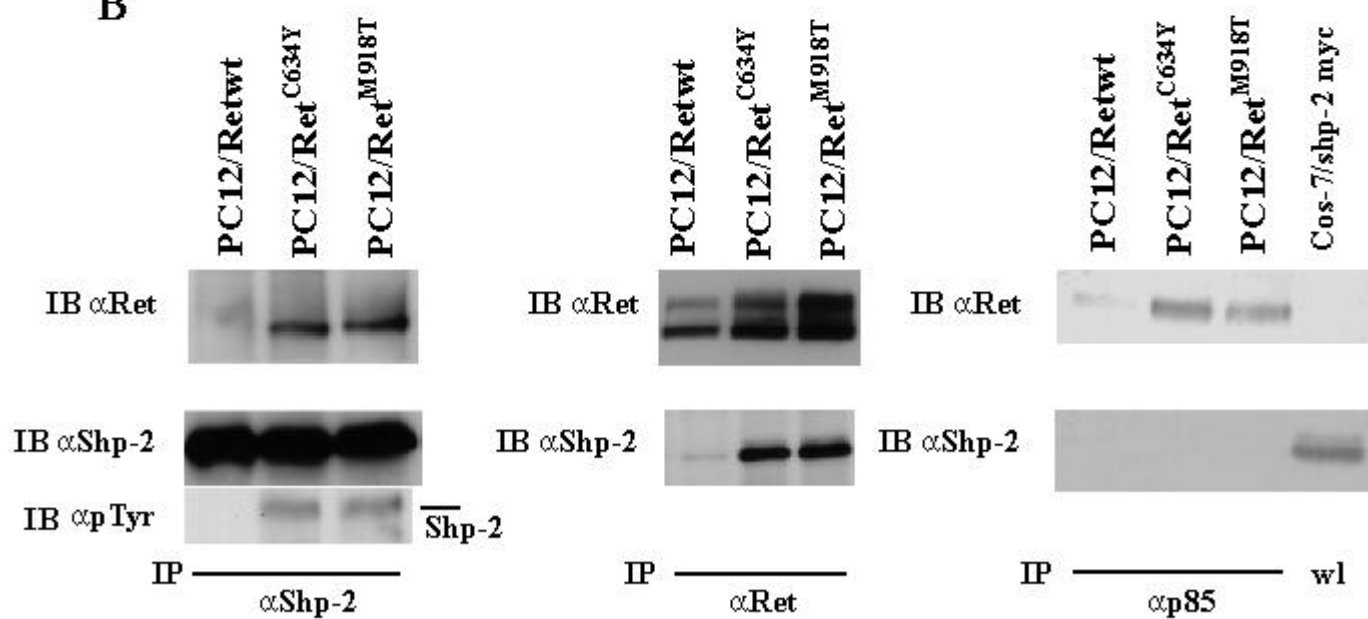
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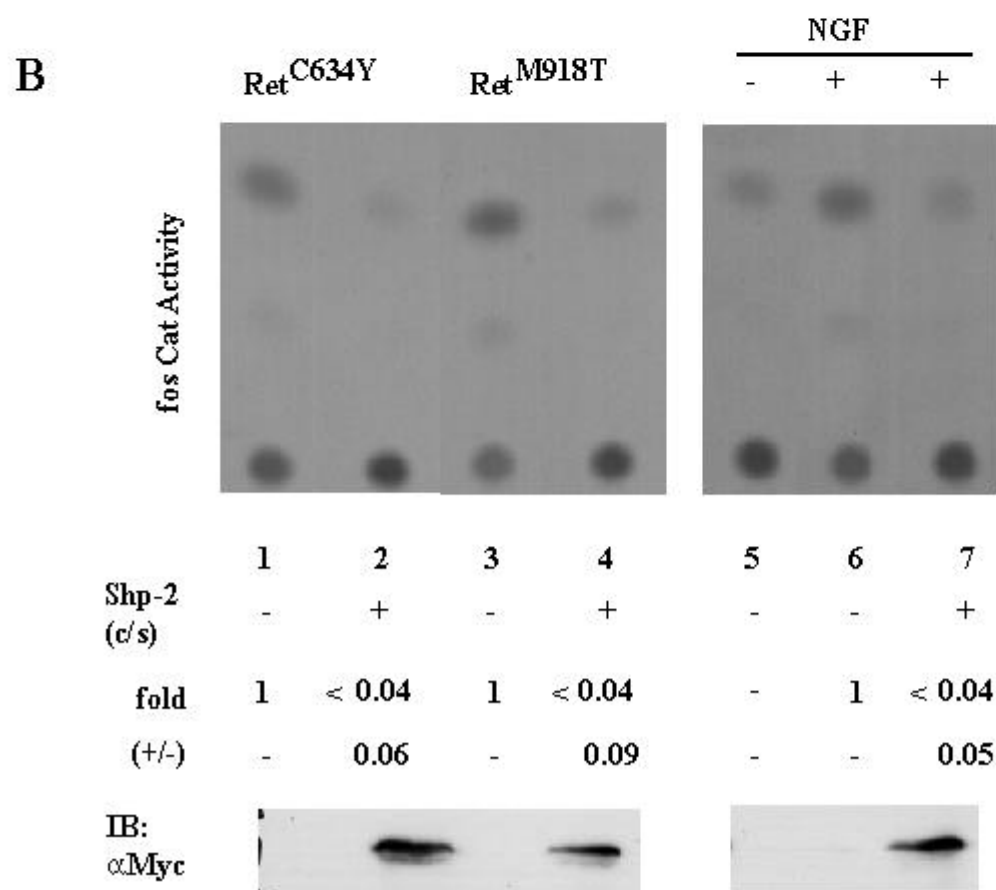
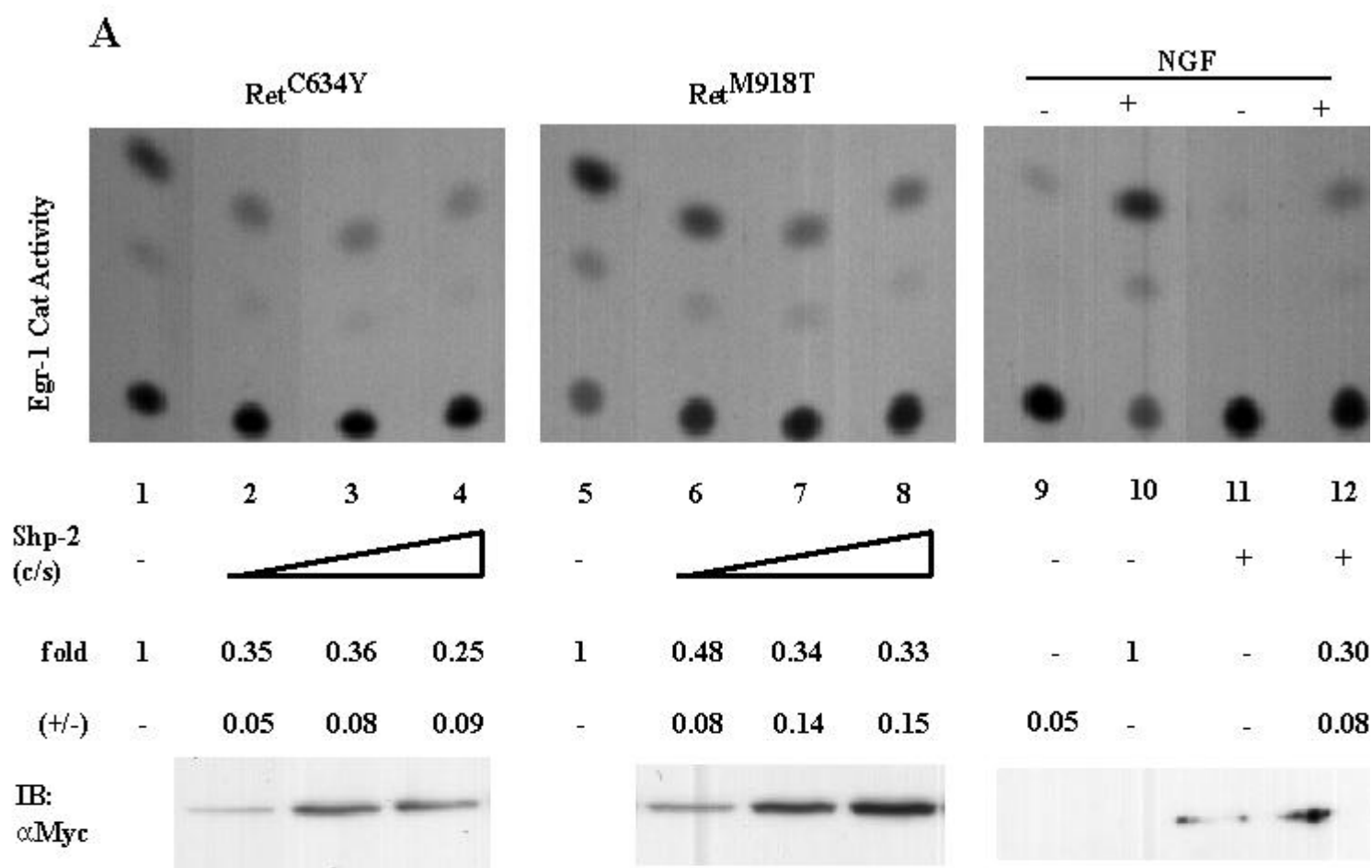


A

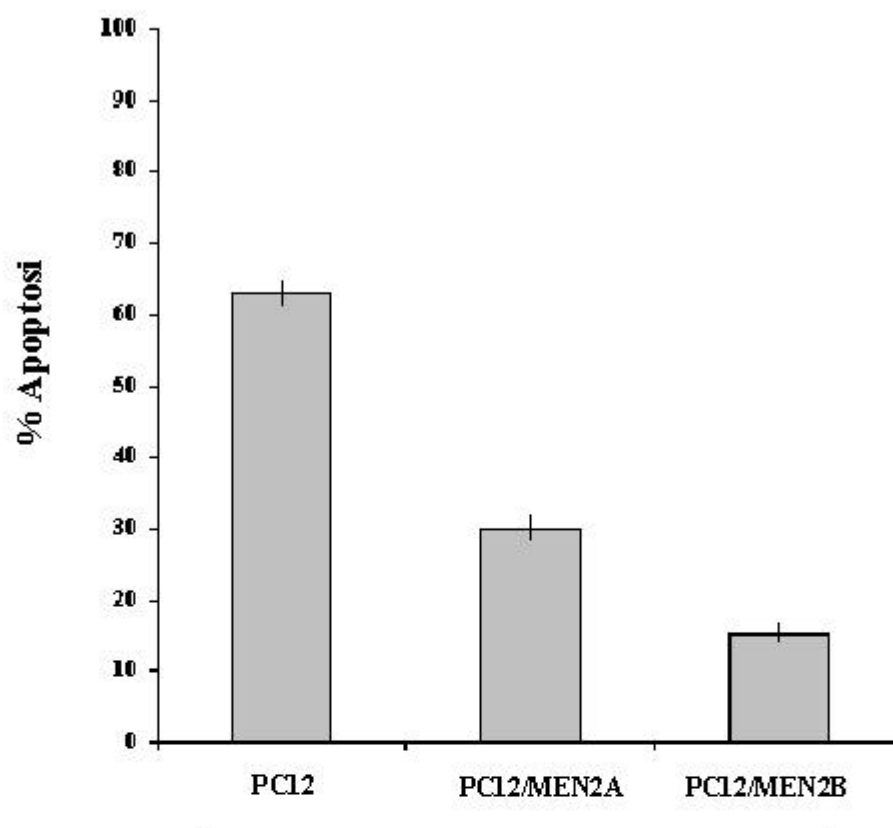


B





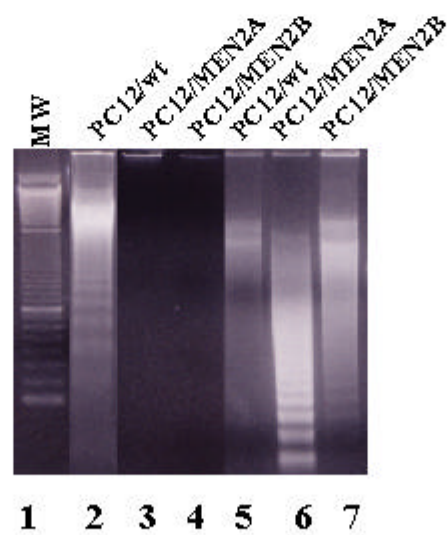
A



**Tempo di crescita
in assenza di siero**

16h

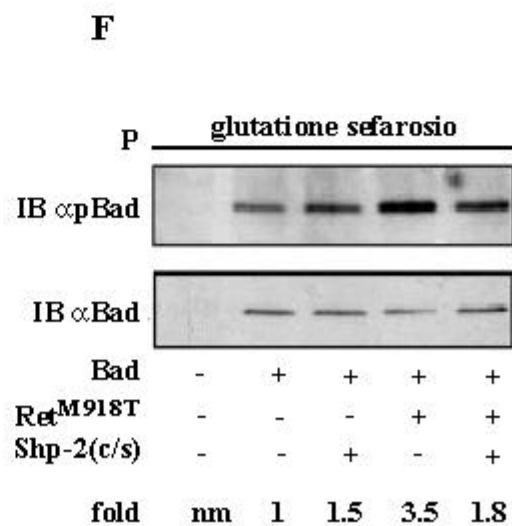
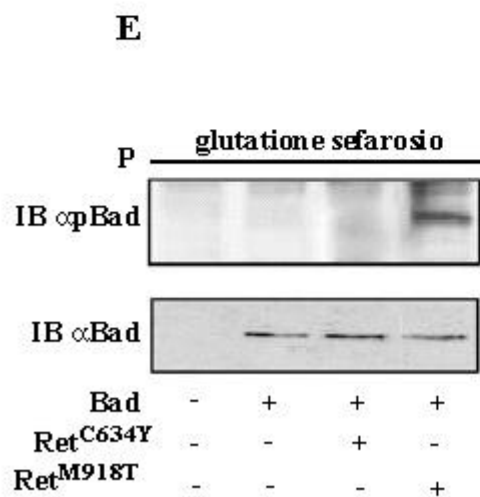
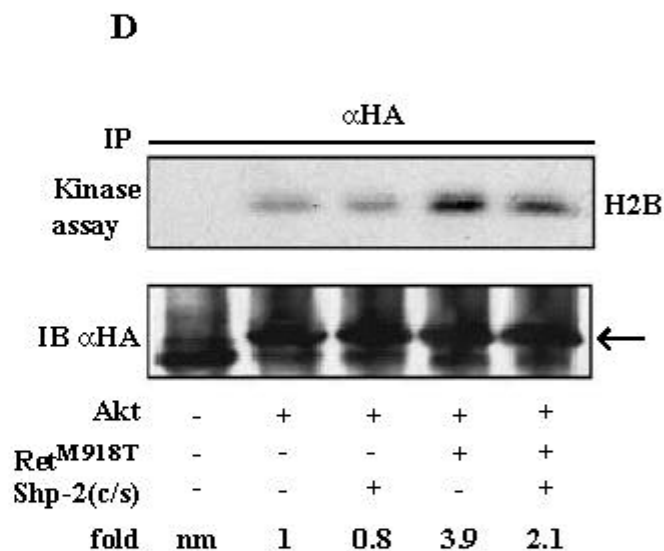
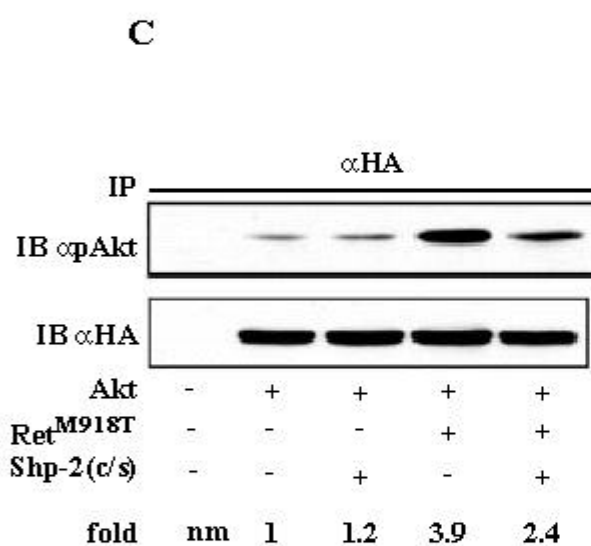
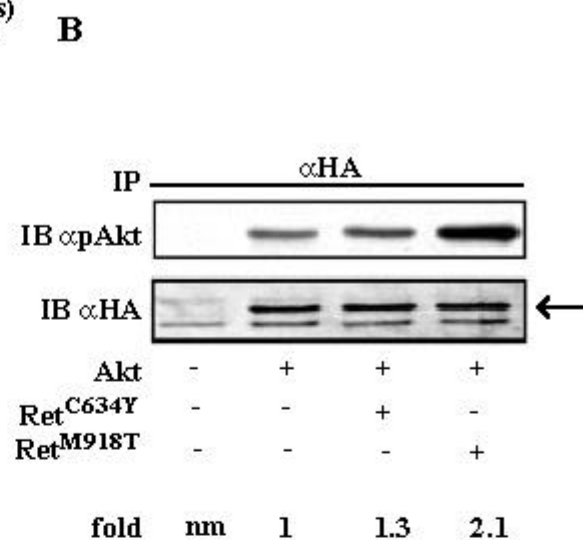
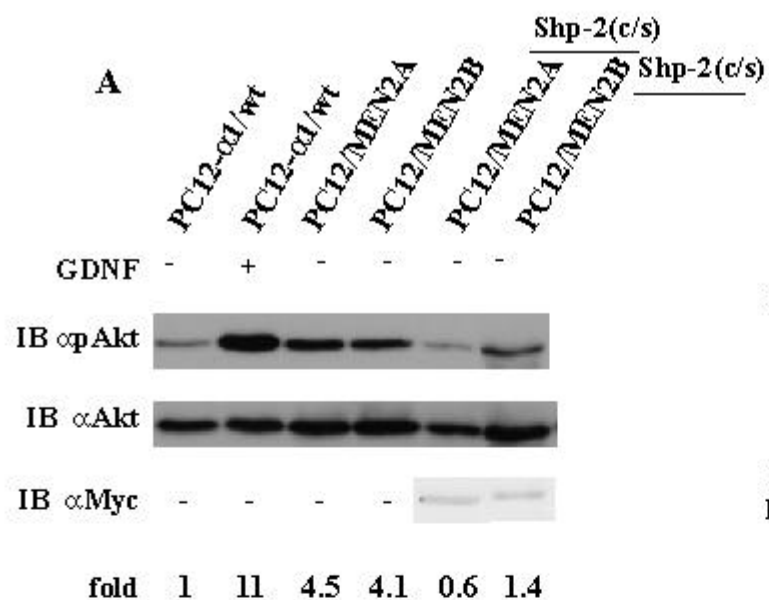
B



**Tempo di crescita
in assenza di siero**

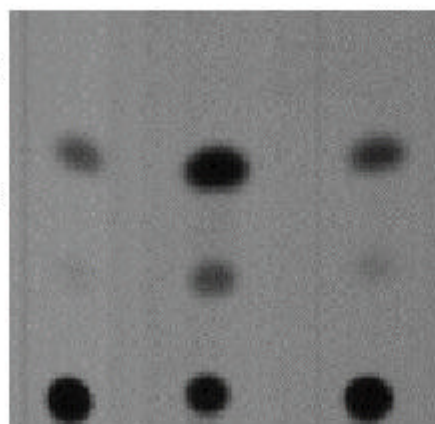
16h

40h

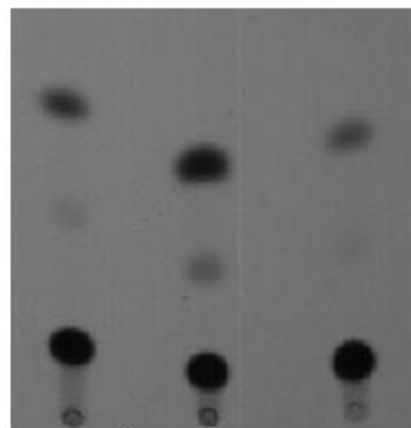


A

Attività del reporter Egr-1 Cat



Attività del reporter fos Cat



Shp-2 (c/s)

-

-

+

GDNF

-

+

+

fold

-

1

0.2

-

-

+

-

+

+

-

1

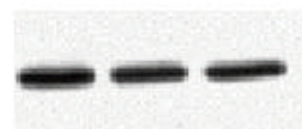
< 0.04

B

IB α pAKT



IB α HA



IP α HA

Shp-2 (c/s)

-

-

+

GDNF

-

+

+

fold

1

2.8

3.2

Fig. 15 (A) I lisati delle linee cellulari indicate nella figura sono stati immunoprecipitati (IP) con anticorpi anti-Shp-1 (sinistra) e anticorpi anti-Shp-2 (destra) ed immunoblottati (IB) con anticorpi anti-pTyr (a). Il filtro è stato strappato, tagliato e ibridato con anticorpi anti-Ret (b), anti-Shp-2 (c) e anti-Shp-1 (d). (B) I supernatanti (SN) ottenuti dagli immunoprecipitati anti-Shp-1 e anti-Shp-2 sono stati immunoprecipitati per la seconda volta con gli stessi anticorpi. Il prodotto degli immunoprecipitati è stato analizzato con anticorpi anti-Ret. (C) I supernatanti ottenuti dagli immunoprecipitati con anticorpi anti-Shp-1 sono stati immunoprecipitati con anticorpi anti-Shp-2 (sinistra) e i supernatanti ottenuti dagli immunoprecipitati con anticorpi anti-Shp-2 sono stati immunoprecipitati con anticorpi anti-Shp-1 (destra). Il prodotto degli immunoprecipitati è stato analizzato con anticorpi anti-Ret (pannello superiore), anti-Shp-2 (pannello centrale) e anti-Shp-1 (pannello inferiore).

Fig. 16 (A) I lisati cellulari delle linee PC12/Ret^{C634Y} (sinistra) e PC12/Ret^{M918T} (destra) sono stati sottoposti a centrifugazione su gradiente di saccarosio. Le singole frazioni del gradiente sono state analizzate per la presenza di Ret (a), Shp-1 (b), Shp-2 (c) e pTyr (d) utilizzando gli anticorpi specifici per queste proteine. Le proteine legate ai “lipid rafts” sono nelle frazioni 4, 5 e 6, mentre le proteine che non legano il “lipid rafts” sono nelle frazioni dalla 9 alla 12. (B) Il lisato cellulare della linea PC12- α 1/wt, in assenza di stimolazione con GDNF, è stato sottoposto a centrifugazione su gradiente di saccarosio e le singole frazioni del gradiente sono state analizzate come è descritto sopra. (C) La quantità di molecole di RET presenti nelle singole frazioni del gradiente è stata determinata usando il programma NIH-image ed è riportata come percentuale. (D) L’istogramma riporta la distribuzione della fosfatasi Shp-1 nelle frazioni contenenti i “lipid rafts” (rafts) e nelle frazioni che escludono i “lipid rafts” (solubile), tale distribuzione è stata determinata usando il programma NIH-image ed i valori ottenuti sono riportati in percentuale.

Fig. 17 Lisati cellulari di cellule PC12/Ret^{C634Y} (A) e PC12/Ret^{M918T} (B) sono stati sottoposti a centrifugazione su gradiente di saccarosio. Le frazioni che contengono i “lipid rafts” (4, 5 e 6) e le frazioni che non contengono i “lipid rafts” (dalle 9 alla 12 riunite) sono state immunoprecipitate (IP) con anticorpi anti-Shp-1 (pannelli di sinistra) o con anticorpi anti-Shp-2 (pannelli di destra) ed immunoblottate (IB) con anticorpi anti-Ret, anti-Shp-1 e anti-Shp-2. Dove è indicato, sono state effettuate delle diluizioni sequenziali (da 5 a 200 volte) della frazione 9-12 riunite. (C) L’istogramma mostra la quantità relativa delle molecole di RET legate ad Shp-1 presenti nelle frazioni che contengono i “lipid rafts” (rafts) e nelle frazioni che li escludono (solubile). La quantità relativa è espressa in percentuale ed è calcolata usando il programma NIH-image.

Fig. 18 (A, pannello superiore) I lisati delle linee cellulari indicate sono stati sottoposti ad analisi per “Far Western Blotting” usando le proteine di fusione GST-(N+C SH2) Shp-1 (pannello di sinistra), GST-(N+C SH2) Shp-2 (pannello centrale) e GST-Shc/FL (pannello di destra). Canali 1 e 2: cellule PC12- α 1/wt non trattate (-) e trattate (+) con GDNF; canali 3 e 4: cellule PC12/Ret^{C634Y} cresciute in presenza (+) e in assenza (-) di siero; canali 5 e 6: cellule PC12/Ret^{M918T} cresciute in presenza (+) e in assenza (-) di siero. (A, pannello inferiore) I filtri ibridati con le proteine di fusione sono stati strappati, tagliati e ibridati con anticorpi anti-Ret. (B) Esperimenti di GST-pulldown usando le proteine di fusione GST-Shc/FL (canali 1-4), GST-(N+C SH2) Shp-1 (canali 5-8) e GST-(N+C SH2) Shp-2 (canali 9-12). Canali 1, 5 e 9: cellule PC12- α 1/wt non

stimolate; canali 2, 6 e 10: cellule PC12- α 1/wt stimulate con GDNF; canali 3, 7, 11: cellule PC12/Ret^{C634Y}; canali 4, 8 e 12: cellule PC12/Ret^{M918T}.

Fig. 19 Dosaggi di fosfatasi *in vitro*. Gli estratti cellulari di cellule PC12/Ret^{C634Y} e PC12/Ret^{M918T} sono stati immunoprecipitati (IP) con anticorpi anti-Shp-1 (A) e anti-Shp-2 (B). Come è descritto in Materiali e Metodi, gli immunoprecipitati sono stati incubati in un buffer di reazione per 0, 1, 5, 10 e 30 minuti a 30°C. Il prodotto della reazione è stato analizzato usando anticorpi anti-pTyr (pannelli superiori). (A e B, pannelli inferiori) I filtri sono stati immunoblottati (IB) con anticorpi anti-Ret per normalizzare l'esperimento.

Fig. 20 (A) Gli estratti cellulari delle linee PC12/wt, PC12/Ret^{C634Y} e PC12/Ret^{M918T} sono stati immunoprecipitati (IP) con anticorpi anti-Gab2 e immunoblottati con anticorpi anti pTyr (pannello di sinistra). Le proteine con un peso molecolare corrispondente al peso molecolare delle proteine Ret, Gab2 e Shp-2 sono indicate con le frecce. Il filtro è stato strappato, tagliato e immunoblottato con anticorpi anti-Ret, anti-Gab2 e anti-Shp-2 (pannello di destra). **(B)** I lisati delle stesse linee cellulari sono stati immunoprecipitati con anticorpi anti-Shp-2 (pannello di sinistra), anti-Ret (pannello centrale) e anti-p85 (pannello di destra). Le cellule Cos-7/Shp-2 myc sono Cos-7 trasfettate stabilmente con il vettore d'espressione per la proteina Shp-2myc, questa linea cellulare è usata come controllo positivo nel legame di Shp-2 alla subunità p85 di PI3K.

Fig. 21 (A) Cellule PC12 sono state trasfettate con il plasmide pEgr-1-CAT (2 μ g) in presenza di 0,5 μ g del vettore LTR-3 (canali 9 e 12), del vettore pRet^{C634Y} (canali 1-4) e del vettore Ret^{M918T} (canali 5-8). Dove è indicato, le cellule PC12 sono state trasfettate con 2 μ g (canali 2 e 6), 4 μ g (canali 3 e 7) e 8 μ g (canali 4, 8, 11, e 12) del vettore d'espressione per la proteina Shp-2(c/s). Dopo 72 h dalla trasfezione le cellule sono state lisate ed analizzate per l'attività della proteina reporter CAT. Come controllo, dopo 24 h dalla trasfezione, le cellule PC12 sono state stimulate con NGF 100ng/ml (canali 10 e 12). **(B)** Cellule PC12 sono state trasfettate con il plasmide p-fos-CAT (2 μ g) in presenza di 0,5 μ g del vettore vuoto LTR-3 (canali 5- 7), del plasmide pRet^{C634Y} (canali 1 e 2) e del plasmide pRet^{M918T} (canali 3 e 4). Dove è indicato, le cellule PC12 sono trasfettate con 8 μ g del plasmide pShp-2(c/s) (canali 2, 4 e 7). Dopo 72 h dalla trasfezione le cellule sono state lisate ed analizzate per l'attività della proteina reporter CAT. Come controllo, dopo 48 h dalla trasfezione, le cellule PC12 sono state stimulate con NGF 100ng/ml (canali 6 e 7). La quantità crescente della proteina Shp-2(c/s) è stata confermata immunoblottando l'estratto cellulare con anticorpi anti-myc.

Fig. 22 (A) Le cellule PC12, PC12/ Ret^{C634Y} e PC12/Ret^{M918T} sono state cresciute per 16 ore in assenza di siero e la percentuale di apoptosi è stata determinata utilizzando un saggio TUNEL. **(B)** L'estrazione del DNA frammentato, dalle cellule PC12, PC12/ Ret^{C634Y} e PC12/Ret^{M918T}, è stato effettuato dopo 16 h e 40 h dalla crescita delle cellule in assenza di siero.

Fig. 23 (A) Gli estratti citosolici, delle linee cellulari indicate in figura, sono stati analizzati per la presenza della fosforilazione di AKT usando anticorpi anti-pAkt, per la presenza della fosfatasi myc-Shp-2(c/s) usando anticorpi anti-myc e normalizzati usando anticorpi anti-Akt. **(B-F)** Cellule PC12 sono state trasfettate transientemente con i vettori di espressione per le proteine Shp-2 (c/s), Ret^{C634Y} e Ret^{M918T} in presenza dei vettori di espressione per la proteina HA-AKT (B-D) e la proteina GST-Bad (E e F). Le cellule sono cresciute per 8 h in assenza di siero e quindi raccolte. I lisati cellulari sono stati immunoprecipitati (IP) con anticorpi anti-HA ed immunoblottati (IB) con anticorpi anti-pAKT (B e C pannello superiore) oppure sottoposti ad un saggio di chinasi (D, pannello superiore). Gli esperimenti sono stati normalizzati utilizzando anticorpi anti-HA (B-C, pannelli inferiori). I lisati cellulari che contengono la proteina GST-Bad (E e F) sono stati incubati con la resina glutatione sefarosio. Il prodotto dell'incubazione è stato analizzato per la fosforilazione della proteina Bad usando anticorpi anti-pBad-112 e anticorpi anti-pBad-136 (pannelli superiori). Gli esperimenti sono stati normalizzati con anticorpi anti-Bad (E e F, pannelli inferiori). I valori delle proteine AKT e Bad fosforilate sono stati determinati usando il programma NIH-image.

Fig. 24 (A) Cellule PC12- α 1/wt sono state trasfettate con il vettore pEgr-1-CAT (pannello di sinistra) o con il vettore pfos-CAT (pannello di destra) in presenza del vettore d'espressione per la proteina Shp-2(c/s). 24 ore dopo la trasfezione, le cellule sono state stimulate per 48 h con 50ng/ml di GDNF e gli estratti citosolici sono stati saggiati per l'attività della proteina reporter CAT. **(B)** Cellule PC12- α 1/wt sono state trasfettate transientemente con il vettore d'espressione per la proteina HA-AKT in presenza del dominante negativo Shp-2(c/s). 36 ore dopo la trasfezione, le cellule sono state cresciute per 12 ore in assenza di siero e quindi stimulate per 5 minuti con GDNF (100ng/ml). I lisati cellulari sono stati immunoprecipitati (IP) con anticorpi anti-HA e immunoblottati (IB) con anticorpi anti-pAKT (pannello superiore). L'esperimento è stato normalizzato ibridando il filtro con anticorpi anti-HA (pannello inferiore).

The Tyrosine Phosphatase Shp-2 Mediates Intracellular Signaling Initiated by Ret Mutants

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The Src homology 2-containing tyrosine phosphatase, Shp-2, is a crucial enzyme that mediates intracellular signaling and is implicated in cell proliferation and differentiation. Here we investigated the involvement of the Shp-2 tyrosine phosphatase in determining the downstream signaling pathways initiated by the Ret oncogene, carrying either the cysteine 634 to tyrosine or the methionine 918 to threonine substitutions. These mutations convert the receptor tyrosine kinase, Ret, into a dominant transforming protein and induce constitutive activation of its intrinsic tyrosine kinase activity leading to congenital and sporadic cancers in neuroendocrine organs.

Using the PC12, rat pheochromocytoma cell line, as model system, we show that Shp-2 mediates immediate-early gene expression if induced by either of the mutant alleles. Furthermore, we show that Shp-2 activity is required for Ret^{M918T}-induced Akt activation. The results indicate that Shp-2 is a downstream mediator of the mutated receptors Ret^{C634Y} and Ret^{M918T}, thus suggesting that it may act as a limiting factor in Ret-associated endocrine tumors, in the neoplastic syndromes multiple endocrine neoplasia types 2A and 2B. (*Endocrinology* 144: 4298–4305, 2003)

THE RET PROTEIN is a transmembrane receptor of the protein tyrosine kinase family. Four distinct ligands for Ret have recently been identified; they are all polypeptide growth factors belonging to the glial cell line-derived neurotrophic factor (GDNF) family. The biological actions of the GDNF family members are mediated through physical interactions with a multicomponent receptor complex consisting of a common signaling component, the Ret receptor tyrosine kinase, and of one of the four members of the glycosyl-phosphatidylinositol-anchored receptor α family, GFR α 1–4 (1–3). Germline mutations in the *ret* gene are responsible for the inheritance of multiple endocrine neoplasia (MEN) type 2A and 2B syndromes, and of familial medullary thyroid carcinoma (FMTC). MEN-2A and MEN-2B are distinct hereditary neoplastic diseases both characterized by the presence of medullary thyroid carcinomas and pheochromocytomas. MEN-2A also features hyperplasia of parathyroid cells, whereas MEN-2B is a more severe disease, being associated with skeletal abnormalities, ganglioneuromas of the intestinal tract, mucosal neuromas, and characterized by an earlier age of tumor onset (4). Missense mutations that cause the creation of an unpaired cysteine residue in the extracellular domain are the most frequent events in FMTC

and in MEN-2A syndrome. A single point mutation, which results in a threonine for methionine substitution at codon 918 within the Ret catalytic domain, is the most frequent mutation in MEN-2B syndrome. Both classes of mutations convert Ret into a dominant transforming gene and cause constitutive activation of its intrinsic tyrosine kinase activity (5–7).

The evidence that inheritance of specific *ret* mutations predisposes to distinct disease phenotypes, supports the belief that some specific cell types undergo abnormal proliferation depending on the type of Ret activation. Indeed, MEN-2A-associated and MEN-2B-associated Ret mutants differ in their activation mechanisms and substrate specificity (5, 8). However, a description of the molecular mechanism(s) responsible for the specificity of each of these diseases (MEN-2B *vs.* MEN-2A) is still lacking (6, 7).

Phosphotyrosine phosphatases constitute a family of regulators (either negative or positive) in the intracellular pathways that result in growth factor-specific cell responses, which act to dephosphorylate signaling intermediaries thereby regulating their function. So far, little is known about the involvement of tyrosine phosphatases as effectors involved in Ret-induced intracellular signaling (9–11).

Shp-2 (previously known as PTP1D, PTP2C, SH-PTP2, SH-PTP3, Syp) is a nontransmembrane phosphotyrosine phosphatase that contains, beside a central catalytic domain, two Src homology 2 domains at the amino terminus and two tyrosine residues located at the carboxy terminus that likely regulate its catalytic activity (12). Shp-2 positively regulates ERKs signaling in response to insulin, fibroblast growth fac-

Abbreviations: CAT, Chloramphenicol acetyl transferase; FMTC, familial medullary thyroid carcinoma; GDNF, glial cell line-derived neurotrophic factor; GFR α 1–4, glycosyl-phosphatidylinositol-anchored receptor α 1–4 family; GST, glutathione-S-transferase; HA, hemagglutinin; MEN, multiple endocrine neoplasia; NGF, nerve growth factor; Ret, receptor tyrosine kinase; Shp2, Src homology 2-containing tyrosine phosphatase; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

tor, and epidermal growth factor, and serves as signal transducer for several receptor tyrosine kinases, G protein-coupled and cytokine receptors (12–19). Besides its implication as positive modulator of the Ras/Erk pathway, Shp-2 has been reported to be necessary for Akt activation by several growth factors (20, 21). Furthermore, as recently reported, Shp-2 differently regulates the kinetics and magnitude of signaling cascades initiated by epidermal growth factor receptor or by other tyrosine kinase receptors, this regulation being receptor specific (22).

Recent evidence in a motoneuron cell line, MN1, implicates Shp-2 as a potential key molecule in Ret signaling. Indeed, upon stimulation of Ret^{wt}, the phosphatidylinositol 3-kinase mediates Ret signaling through a multiprotein complex that includes the docking proteins Gab2 and Shp-2 (9). In this report, we investigated the implication of Shp-2 in determining the intracellular signaling initiated by two different Ret mutants (either associated to MEN-2A or MEN-2B syndromes). The results presented here indicate that, in PC12 cells, Shp-2 activity is required for both Ret mutants signaling but mediates distinct functions according to which Ret mutant is involved.

Materials and Methods

Cell culture and transfections

Parental PC12 cells, PC12/wt, PC12/MEN2A, PC12/MEN2B, and PC12- α 1/wt cell lines, which expressed Ret^{wt}, Ret^{C634Y}, Ret^{M918T}, and Ret^{wt} plus GFR α -1 proteins, respectively, were cultured as previously described, and when required, with the appropriate selection pressure (23). The PC12/MEN2A and PC12/MEN2B cells were transfected with myc-SH-PTP2 (C/S) plasmid and selected for 15 d in presence of 400 ng/ml of G418 to obtain PC12/MEN2A^{Shp-2 C/S} and PC12/MEN2B^{Shp-2 C/S} cells, respectively. A mass population and individual clones were grown and maintained in selection medium.

The pEBG-mBad comprises the sequence relative to Bad gene fused with glutathione-S-transferase (GST) gene sequence (New England Biolabs, Inc., Beverly, MA). The pCEFL-HA-Akt, contains the Akt gene fused to the hemagglutinin (HA) epitope. Plasmids encoding myc-SH-PTP2 or myc-SH-PTP2 (C/S) were kindly provided by Dr. J. Pessin (University of Iowa, Iowa City, IA). The pfos-CAT (–356 to +109) (24), pEgr-1-CAT (C4) (–1150 to +200) fused to the chloramphenicol acetyl transferase (CAT) gene (23).

Preparation of cell extracts, immunoprecipitation, and immunoblotting

Between 10^6 and 10^7 cells were washed twice in ice-cold PBS, then lysed in lysis buffer [50 mM Tris-HCl (pH 8) containing 150 mM NaCl, 1% Nonidet P-40, 2 μ g/ml aprotinin, 1 μ g/ml pepstatin, 2 μ g/ml leupeptin, 1 mM Na₃VO₄]. Protein concentrations were estimated by a modified Bradford assay (Bio-Rad, Hercules, CA). One milligram of total cell lysate was incubated with specific antibodies for 2 h at 4 C and then immunoprecipitated with protein G-plus agarose (Calbiochem, Oncogene Research) overnight at 4 C. Immunoprecipitates were washed five times with the above lysis buffer and boiled in Laemmli buffer for 5 min, then subjected to SDS-PAGE (10% acrylamide) and transferred to polyvinylidene difluoride membrane (Millipore Co., Bedford, MA). The antibodies used were: anti-SH-PTP2 (C-18), anti-RET (C-19), and anti-myc (9E10) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-Gab2 and antiphosphotyrosine monoclonal antibodies (4G10) from Upstate Biotechnology Inc. (Lake Placid, NY); anti-HA from Roche Molecular Biochemicals (Basel, Switzerland); anti-pAkt (Ser473), anti-Bad, anti-pBad-112, and anti-pBad-136 primary antibodies from New England Biolabs Inc., and detected with the ECL system (Amersham Biosciences Corp., Piscataway, NJ). When indicated, filters were stripped in 62.5 mM Tris-HCl (pH 6.7), 100 mM 2-mercaptoethanol, 2% sodium

dodecyl sulfate, for 30 min at 55 C. The immunoblots shown are examples of at least three independent experiments.

DNA fragmentation analysis and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay

For the extraction of fragmented DNA, 2.5×10^6 cells/sample were incubated in serum-free culture medium for 16 h and 40 h (as indicated), then harvested and lysed in a buffer containing 0.5% Triton X-100, 5 mM Tris-HCl (pH 7.4), 20 mM EDTA. Intact nuclei were removed by centrifugation, and soluble DNA was purified by phenol extraction and ethanol precipitation. Soluble DNA was analyzed by electrophoresis on a 1.2% agarose gel. An equal number of cells was subjected to TUNEL assay following the manufacturer's instructions (Roche Molecular Biochemicals). Apoptosis was evaluated by Fast Red (Dako Co., Carpinteria, CA) staining.

GST capture

For GST capture, experiments cells were harvested 48 h after transfection, then GST-Bad fusion proteins were purified by incubating 1 mg of cell extracts with glutathione Sepharose (Amersham Biosciences Corp.) for 2 h at 4 C, subjected to SDS-PAGE and immunoblotted.

CAT assay

Cell extracts were harvested 72 h after transfection, and CAT activity was analyzed by thin-layer chromatography, as previously described (23). The individual spots were isolated and counted in a scintillation counter. For each experiment, the percentage of conversion to acetylated chloramphenicol ¹⁴C was then calculated and normalized for the transfection efficiency. Equal transfection efficiency was confirmed for each experimental point by cotransfection with the pSV-Luc reporter plasmid, and measuring of the luciferase activity.

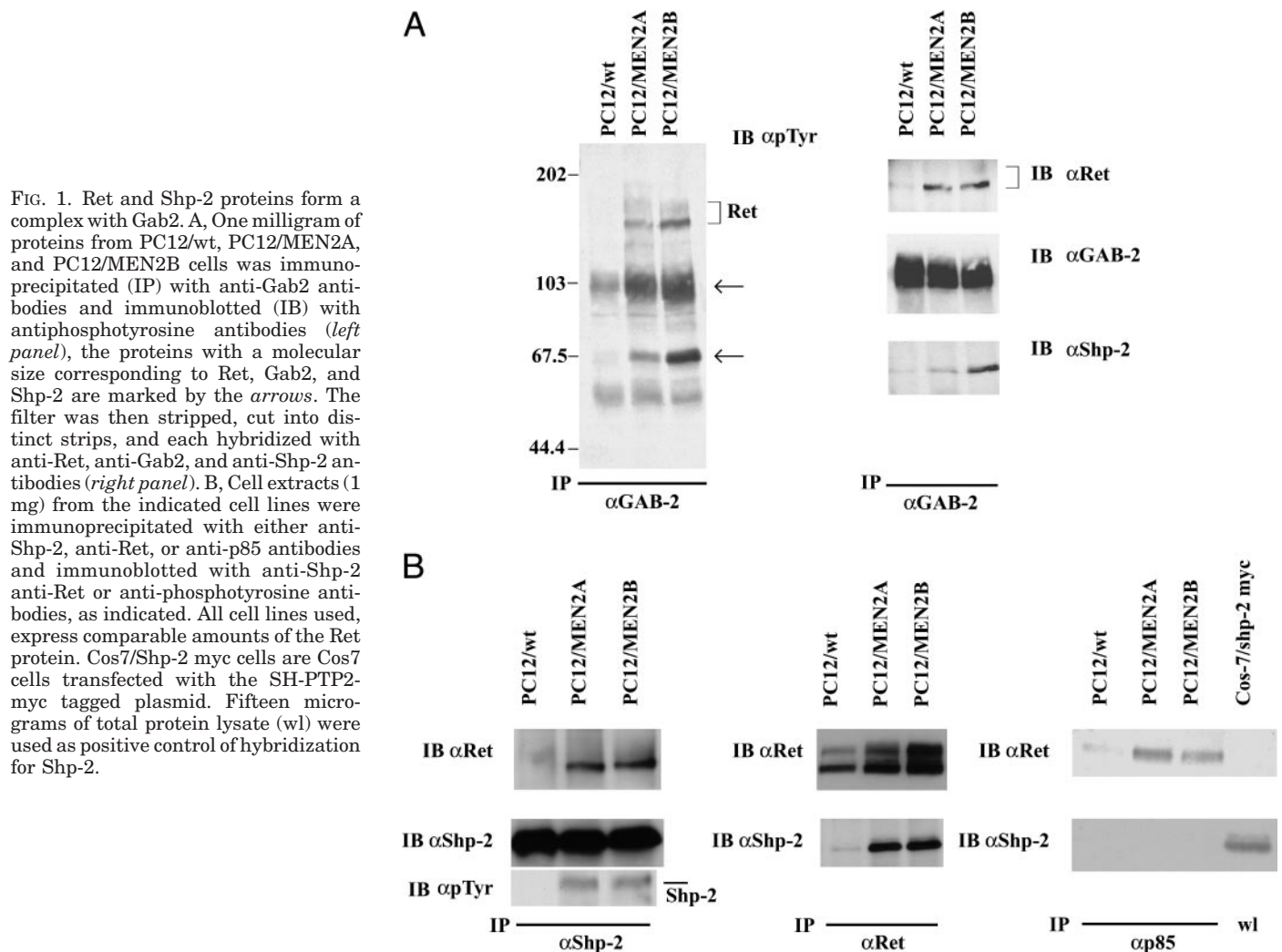
Immunocomplex Akt kinase assay

To appreciate the effects of the Shp-2 (C/S) we transfected PC12 cells at low molar ratio (Ret:Akt = 1:6). These conditions were sufficient to observe the stimulation of pAkt, still enabling to observe the interfering action of Shp-2 (C/S). In these conditions, phosphorylation of Akt was stimulated up to 4-fold over the background by Ret^{M918T} and less than 1.5-fold by Ret^{C634Y}. Forty-eight hours after transfection, cells were harvested, and 500 μ g of total cell lysate were immunoprecipitated with anti-HA antibody for 2 h at 4 C. Samples were washed three times with kinase buffer [20 mM HEPES (pH 7.4), 10 mM MgCl₂, 10 mM MnCl₂], and incubated in the same buffer containing 60 μ g/ml histone 2B from Upstate Biotechnology Inc. (Lake Placid, NY), 1 mM ATP, 1 mM dithiothreitol, and 10 μ Ci of [α -³²P]ATP for 30 min at 25 C and stopped with Laemmli buffer. The expression of Ret mutants upon transient transfections was confirmed indirectly by performing parallel assays of Egr-1-CAT activity. The products of the kinase reactions were separated on SDS-PAGE, blotted, and autoradiographed.

Results

Both Ret^{C634Y} and Ret^{M918T} form a complex with Shp-2

Given that GDNF may induce the formation of a multiprotein stable complex in which Shp-2 recruitment to Ret is mediated by Gab2 (9), we first examined whether similar interactions might take place between constitutively active Ret mutants (Ret^{C634Y} or Ret^{M918T}) and Shp-2. To this aim, we used PC12-derived cell lines stably transfected with either Ret^{wt}, Ret^{C634Y} and Ret^{M918T} alleles (PC12/wt, PC12/MEN2A, and PC12/MEN2B, respectively). These cell lines express comparable levels of the Ret, Shp-2, and Gab2 proteins (Ref. 25 and Fig. 1). Cell extracts were immunoprecipitated with anti-Gab2 antibodies and analyzed



by immunoblotting with anti-pTyr, anti-Ret and anti-Shp-2 antibodies. As shown (Fig. 1A, right upper panel), the active Ret proteins (either Ret^{C634Y} or Ret^{M918T}) and Shp-2 were both immunoprecipitated together with Gab2. On the other hand, the unstimulated Ret^{wt} did not coimmunoprecipitate with Gab2 (first lane). As determined by hybridizing with antiphosphotyrosine antibodies such complex consists of several tyrosine phosphorylated proteins, three of which can be identified as Ret, Shp-2, and Gab2 based on the respective molecular sizes (Fig. 1A, left panel). Furthermore Shp-2 and the active Ret mutants, but not the wild-type inactive Ret, were found in the same immunocomplex (Fig. 1B), though, Gab2-Ret^{C634Y} complex binds Shp-2 at a much lesser extent compared with Gab2-Ret^{M918T} (Fig. 1A, right panel). On the other hand, the active mutant Ret proteins, but not Shp-2, coimmunoprecipitate with the p85 regulatory subunit of the PI3Kinase, that has been previously described to be recruited to the active Ret complex. Consistently with previous reports, our results indicate that active Ret is functionally coupled to tyrosine phosphorylated Shp-2, and interaction is likely mediated by Gab2 but not by p85. Whether Ret is itself substrate of Shp-2, and whether molecular partners, other than Gab2,

mediate the interactions of Shp-2 with Ret^{C634Y} is under investigation.

Shp-2 is involved in Ret mutants-induced PC12 cell differentiation

We investigated the functional relevance for intracellular signaling of Shp-2 recruitment by Ret (either Ret^{C634Y} or Ret^{M918T}). In PC12 cells, the active Ret mutants are able to induce the transcription of immediate early genes involved in neuronal differentiation, including *Egr1* and *c-fos*. Indeed, both Ret^{M918T} and Ret^{C634Y} mutant proteins induce at similar extents the *Egr1* promoter and such induction is inhibited by a dominant interfering mutant of Ras (23, 25). Thus, to investigate the role of Shp-2 in Ret-induced signaling, we first examined the effect on *Egr1* and *c-fos* promoters induction of the interfering mutant Shp-2 C459S (C/S). This mutant is still able to bind substrates, but it is catalytically inactive.

Thus, we used an *Egr1*-CAT plasmid in which the CAT expression was driven by the *Egr1* promoter. We cotransfected PC12 cells with increasing amounts of an expression construct for Shp-2 together with a construct for Ret^{C634Y}. Transfecting Shp-2 (wt) did not induce any relevant change

of the CAT activity (not shown). In contrast, the Shp-2 (C/S) mutant inhibited the *Egr-1* and *c-fos*-promoter dependent CAT activity (reaching up to 75% of inhibition for the *Egr-1* and more than 95% for the *c-fos* promoter) (Fig. 2A, lanes 2–4; and B, lanes 1 and 2). As shown in Fig. 2, the extent of inhibition of Ret^{C634Y} effects was similar to that observed

following nerve growth factor (NGF) stimulation of *Egr-1*-CAT (lanes 10 and 12). The persistence of CAT activity in the presence of maximal amounts of the Shp-2 (C/S) mutant suggests that, even though this phosphatase contributes to the positive regulation of downstream signaling, Shp-2-independent pathways are also relevant for *Egr1* (but not

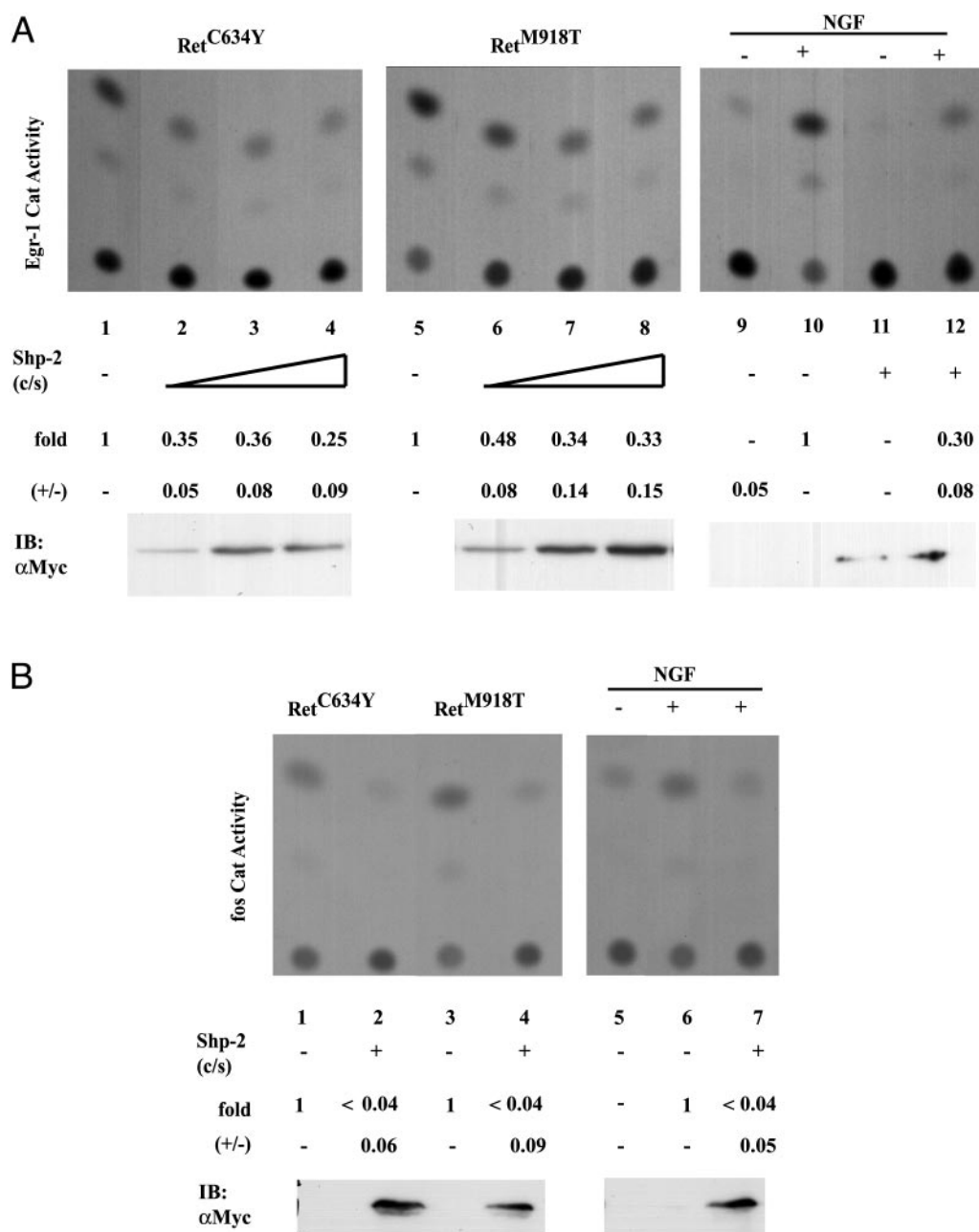


FIG. 2. Shp-2 is required for *Egr-1* promoter induction. A, PC12 cells were transfected with pEgr-1-CAT (2 μ g) together with 0.5 μ g of LTR-3 vector (lanes 9–12), or pRet9^{C634Y} (lanes 1–4) or pRet9^{M918T} (lanes 5–8). Where indicated, cells were also cotransfected with 2 μ g (lanes 2 and 6), 4 μ g (lanes 3 and 7) and 8 μ g (lanes 4, 8, 11, and 12) of a plasmid encoding Shp-2 (C/S) mutant. As a control, NGF (100 ng/ml) was added 24 h after transfection (lanes 10 and 12). B, PC12 cells were transfected with pfos-CAT (2 μ g) together with 0.5 μ g of LTR-3 vector (lanes 5–7), or pRet9^{C634Y} (lanes 1 and 2) or pRet9^{M918T} (lanes 3 and 4). Where indicated, cells were also cotransfected with 8 μ g (lanes 2, 4, and 7) of a plasmid encoding Shp-2 (C/S) mutant. As a control, NGF (100 ng/ml) was added 24 h after transfection (lanes 6 and 7). In all experiments, total transfected DNA was kept constant by adding increasing amounts of the empty vector. Increasing amounts of the Shp-2 protein were confirmed by immunoblot with anti-myc antibody. The fold values of CAT activity in the presence of Shp-2 (C/S) mutant have been calculated over the control (in the absence of phosphatase) set to 1 (Fig. 2A, lanes 1, 5, and 10; and Fig. 1B, lanes 1 and 6). The results represent an example of three separate transfections performed in duplicate; the results of individual transfection varied by less than 25%.

c-fos) promoter induction by these tyrosine kinase receptors. Upon transfection of the Ret^{C634Y} together with Shp-2 (C/S), NGF stimulation did not restore the levels of Egr-1-CAT activity, thus indicating that NGF stimulates Shp-2-dependent common substrates (not shown).

Because the methionine 918 to threonine substitution causes a change in substrate specificity of the Ret kinase, we determined whether Shp-2 might be implicated in signal transmission through the Ras/Erk cascade (as assessed by the induction of the Ras-dependent Egr-1 promoter induction) even if initiated by the Ret^{M918T} mutant. To this aim, we transfected PC12 cells with increasing amounts of Shp-2 expression vectors together with the Ret^{M918T}. As shown in Fig. 2, the Shp-2 (C/S) mutant, but not Shp-2 (wt) (not shown), inhibited Egr-1- (Fig. 2A, lanes 6–8) and *c-fos*-CAT (Fig. 2B, lanes 3 and 4) activity induced by Ret^{M918T}. These results indicate that Shp-2 mediates the immediate early *Egr1* and *c-fos* promoter-induction by either the Ret^{C634Y} and the Ret^{M918T} mutants.

Shp-2 mediates Ret^{M918T}-induced cell survival

Given the importance of the PI3K/Akt signal cascade for the transforming activity of Ret (26), we asked whether binding of Shp-2 might be involved in the induction of survival signals triggered by Ret mutants. In agreement with previous reports (26, 27), expressing either Ret^{C634Y} or Ret^{M918T} oncogenes protects PC12 cells by trophic withdrawal-induced apoptosis. Using PC12/MEN2A and PC12/MEN2B cell lines

we show that the expression of Ret^{M918T} rescued PC12 and PC12/wt cells from apoptosis more efficiently compared with Ret^{C634Y}, and further delayed the genomic DNA fragmentation (Fig 3). Because Ret^{C634Y} protects PC12 cells from serum withdrawal-mediated apoptosis by activating the PI3K/Akt cascade (27), we determined whether Shp-2 activity might be involved in the activation of this pathway. To this aim, we generated stable PC12/MEN2A and PC12/MEN2B derived-cell lines that stably express the Shp-2 (C/S) and analyzed the levels of Akt Ser 473 phosphorylation, as indicative of its activation (28). To this aim, we took advantage of a PC12/wt derivative cell line that expresses GFR α 1 (PC12- α 1/wt). In these cells, GDNF causes activation of Ret^{wt} kinase and neurite outgrowth (data not shown). As shown in Fig. 4A, in PC12- α 1/wt cells GDNF rapidly induces phosphorylation of endogenous Akt, similarly, in both PC12/MEN2A and PC12/MEN2B cells, Akt phosphorylation was constitutive, although at reduced extent compared with acute stimulation. On the other hand, stable expression of Shp-2 (C/S) in PC12/MEN2A^{Shp-2 C/S} and PC12/MEN2B^{Shp-2 C/S} strongly reduced phosphorylation of Akt compared with parental cells (PC12/MEN2A and PC12/MEN2B cells, respectively), even though the extent of inhibition largely varied among all the clones analyzed (not shown; and *Materials and Methods*). Therefore, to further confirm the implication of Shp-2 in Akt activation and to avoid possible artifacts caused by the clonal expansion of stable Shp-2 (C/S) transfectants, we performed transient transfections in PC12 cells. However,

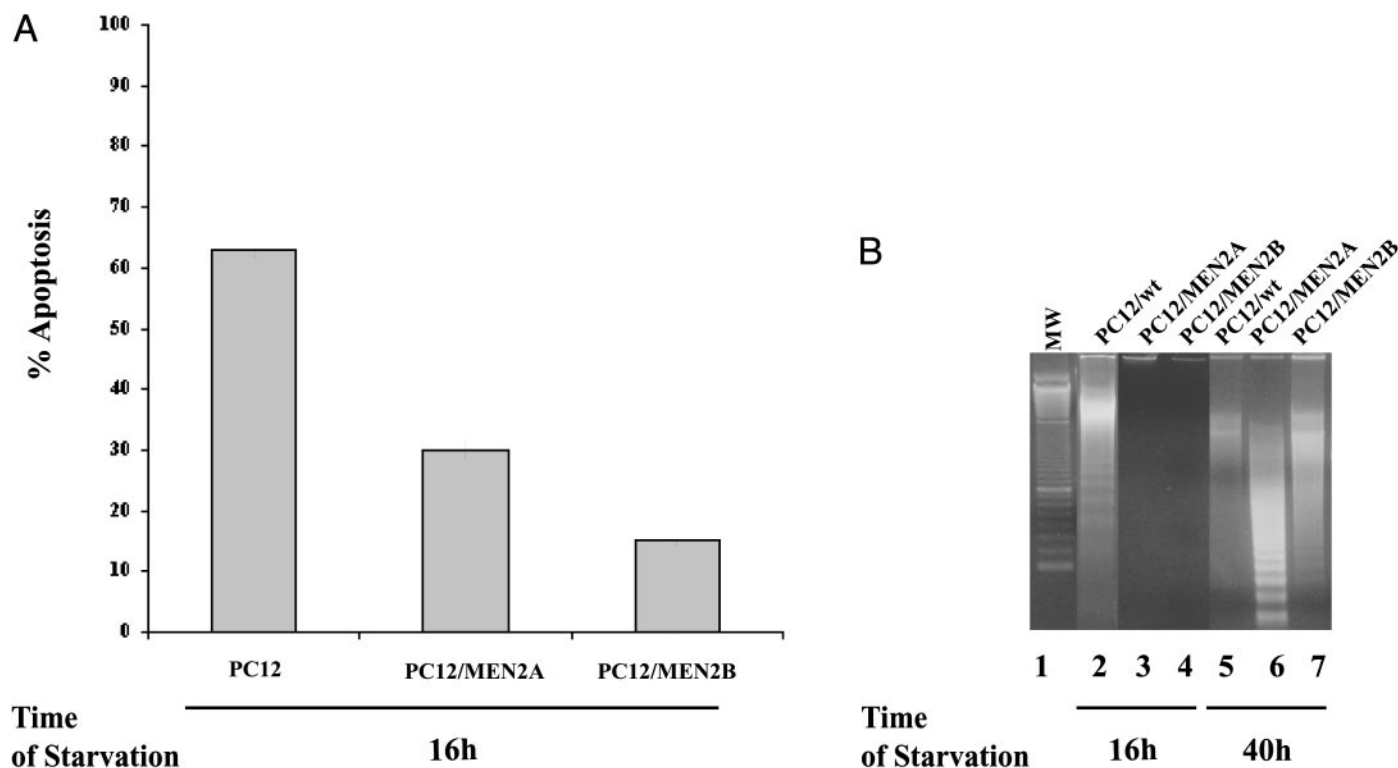


FIG. 3. PC12/MEN2A and PC12/MEN2B cells resist to serum starvation at different degrees. A, PC12, PC12/MEN2A, and PC12/MEN2B cells were serum starved for 16 h, and the percent of apoptosis was determined by TUNEL assay. The bar graphs indicate values scored from six different frames analyzed (with at least 20 cells analyzed per frame). B, Soluble DNA was extracted from PC12/wt, PC12/MEN2A, and PC12/MEN2B cells as indicated starved for 16 and 40 h.

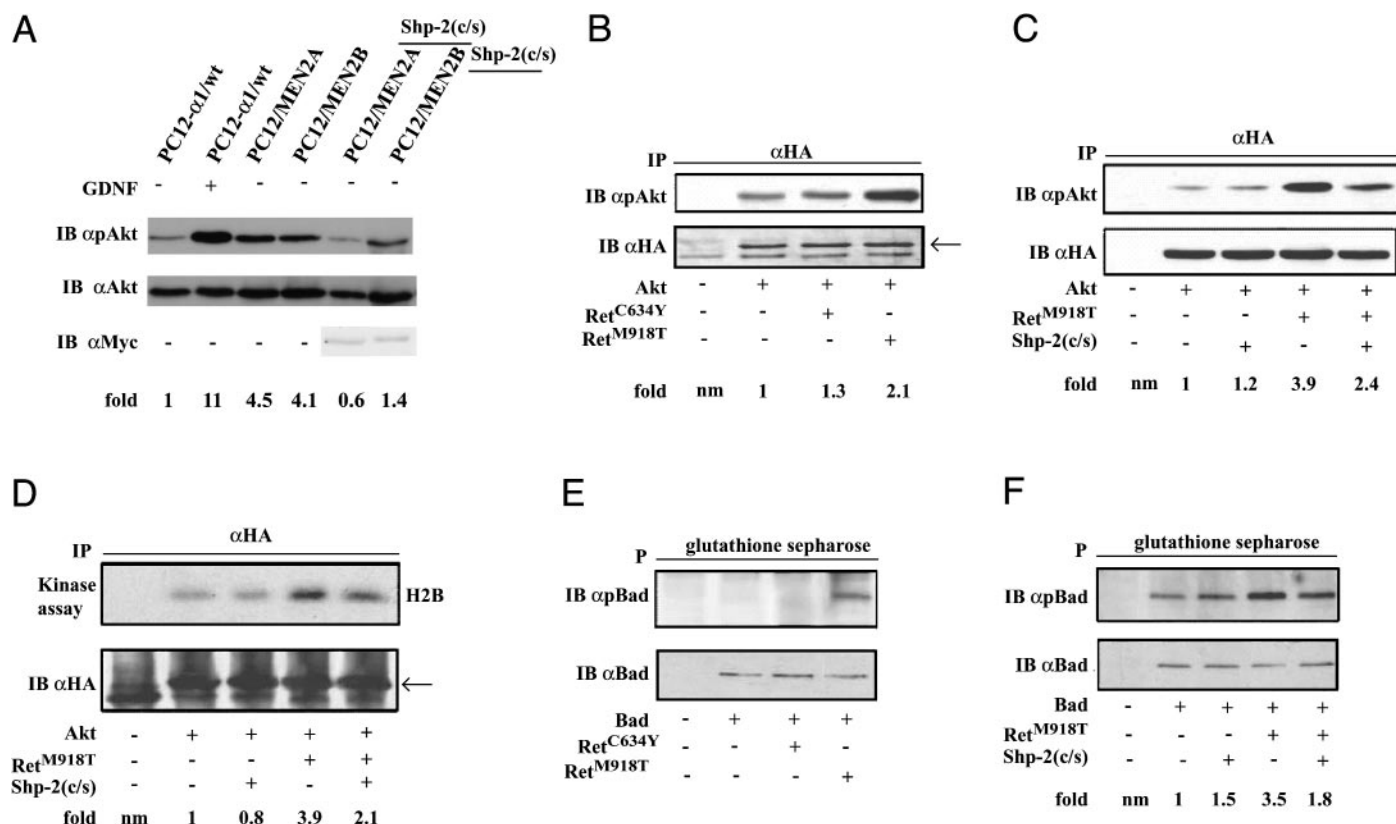


FIG. 4. Shp-2 is involved in cell survival. A, Equal amounts of total protein extracted from PC12- $\alpha 1$ /wt cells either untreated or treated with GDNF (50 ng/ml), PC12/MEN2A, PC12/MEN2B, PC12/MEN2A^{Shp-2 C/S}, PC12/MEN2B^{Shp-2 C/S} cells (as indicated) were analyzed by immunoblot with anti-pAkt, anti-Akt and anti-Myc antibodies. B–F, PC12 cells were transiently transfected with Shp-2 C/S plasmid, pRet9^{C634Y} or pRet9^{M918T} (as indicated) together with either HA-Akt (B–D) or with GST-Bad plasmids (E and F). Cells were kept 8 h in serum free medium then harvested. Cell lysates were immunoprecipitated (IP) with anti-HA antibody and immunoblotted (IB) with anti-pAkt (B and C, upper panel), or subjected to immuno kinase assay (D, upper panel). The amount of immunoprecipitated HA-Akt protein was checked by immunoblot of the same membrane with anti-HA antibodies (B–D, lower panel). After purification GST-Bad proteins were immunoblotted with a mixture of anti-pBad¹¹² and -pBad¹³⁶ antibodies (E and F, upper panel), or single antibodies (not shown). Same filter was hybridized with anti-Bad antibodies (E and F, lower panel). Similar results were obtained by immunoblot of total cell lysates (not shown). The values of pAkt and pBad induction in the presence of Ret or Ret mutants have been calculated using the NIH Image Program, as fold increase over the control (in the absence of Ret stimulation) set to 1, except for panel E, in which the control was not measurable. nm, Not measurable.

because only a fraction of cells became transfected (approximately 10% as determined with a GFP expressing vector, not shown), any interference of the exogenous Shp-2 (C/S) on the overall extent of phosphorylation of endogenous Akt could not be appreciated. Therefore, we transfected the PC12 cells with a reporter construct for HA-Akt together with that expressing the mutated Ret protein (either Ret^{C634Y} or Ret^{M918T}), and analyzed the levels of Akt Ser 473 phosphorylation. Furthermore, to better appreciate the inhibitory effects of the Shp-2 (C/S) we performed all experiments in nonsaturating conditions of Ret stimulation (*Materials and Methods*). As shown in Fig. 4B, phosphorylation of Akt was clearly stimulated by Ret^{M918T}, but remained low upon expression of Ret^{C634Y}, indicating that this pathway is preferentially activated by the Ret protein carrying the methionine 918 to threonine mutation. Thus, to investigate the involvement of Shp-2 in Ret^{M918T} signaling we induced Akt activation by expressing Ret^{M918T} together with the catalytically inactive Shp-2 (C/S) phosphatase. Expression of Shp-2 (C/S) strongly reduced both phosphorylation and kinase activity of Akt (Fig. 4, C and D).

Because phosphorylation by Akt causes Bad (a proapoptotic member of the Bcl-2 family) to dissociate from Bcl-2 and thus makes it unable to promote cell death (28), we checked the induction of Bad phosphorylation by both Ret mutants. As expected, expressing Ret^{M918T} induced Bad phosphorylation (on both Ser112 and Ser136) (Fig. 4E), and this phosphorylation was strongly inhibited by the Shp-2 (C/S) mutant (Fig. 4F). Though, in PC12/MEN2B and in PC12/MEN2A, Akt activity is constitutive and requires continuous activation of Shp-2, these results indicate that expressing Ret^{M918T}, and barely Ret^{C634Y}, rapidly induces activation of the Akt/Bad pathway. Taken together, our results indicate that Shp-2 is likely required to protect PC12 cells from serum withdrawal-mediated apoptosis.

Shp-2 mediates GDNF-induced signaling

To further support the implication of Shp-2 in Ret signaling, we addressed the question of the involvement of Shp-2 in GDNF induced intracellular signaling. To this aim, we took advantage of a PC12- $\alpha 1$ /wt cells. In these cells, GDNF

stimulates immediate early gene expression and phosphorylation of endogenous Akt several times (Fig. 4A and data not shown). As shown in Fig. 5A, transfecting the Shp-2 (C/S) mutant inhibited both the *Egr-1* and *c-fos* promoter-dependent CAT activity.

Further, we tested whether the stimulation of the PI3K/Akt cascade by GDNF was mediated by Shp-2. To this aim, we made transient transfections of PC12- α 1/wt cells with an expression plasmid for HA-Akt and analyzed the levels of Akt Ser 473 phosphorylation. As shown in Fig. 5B, upon 48 h of stimulation with GDNF, the extent of HA-Akt phosphorylation was around three times over basal, thus confirming that in these cells GDNF stimulates the PI3K pathway. However, in contrast to that observed above for the inhibition of the immediate-early promoter induction, expressing Shp-2 (C/S) had no effect on the extent of Akt phosphorylation. These results support the assumption that Shp-2 mediates expression driven by the Erk-dependent of *Egr-1* and *c-fos* promoters, both upon acute stimulation (by GDNF) and upon induction by the active Ret^{C634Y} and Ret^{M918T} oncogenes, but that differently regulates the activation of Akt.

Discussion

Here we report that Shp-2 activity mediates multiple signaling pathways initiated by two mutants of the Ret oncogene, each being responsible for a distinct disease: Ret^{C634Y} causes inheritance of MEN-2A and, rarely, FMTc, whereas Ret^{M918T} causes inheritance of MEN-2B. Indeed, by using stable Ret transfectants (PC12/MEN2B and PC12/MEN2A) first we show that in PC12 cells Shp-2 may be recruited to a complex with the oncogenic active Ret molecules (either Ret^{M918T} or Ret^{C634Y}), but not with unstimulated Ret^{wt}, and likely involving binding to Gab2. The finding that constitutively active Ret molecules may form a complex with Shp-2, although in good agreement with recent reports of GDNF-dependent Shp-2 interaction with the wild-type Ret (9, 29),

looks in contrast with what reported by expressing a different Ret mutant carrying a 9-bp duplication (Ret-9bp) in NIH 3T3 (11). The most plausible interpretation for this discrepancy likely relies on the evidence reported here that binding to Shp-2 would also depend on which Ret mutant allele is involved (*i.e.* binding to Ret^{C634Y} is less efficient compared with Ret^{M918T}). Whether phosphorylation or recruitment of specific Ret substrates may account for these differences is under investigation.

Once recruited to the active Ret, Shp-2 likely interacts with other effector molecules and acts as a positive regulator of downstream signaling. Indeed, in analogy with reports using NGF (30), the induction by Ret of immediate-early gene expression is depressed by the expression of a dominant interfering mutant of Shp-2. Drastic inhibition by the Shp-2 mutant was observed either with GDNF-stimulated Ret^{wt} or with any of the mutant oncogenes. Given that both Ret mutants require the continuous activation of Ras/Erk pathway to induce the expression of the immediate early genes (25), this indicates that activation of this cascade would be mediated by Shp-2, though the extent depends on the Ret genetic background. This is consistent with several reports that implicate the phosphatase activity of Shp-2 in the activation of the Ras/Erk pathway by several receptor tyrosine kinase (16).

On the other hand, as recently reported, Shp-2 acts to regulate strength and duration of PI3K/Akt cascade in a receptor-specific manner (22). In this context, our results, indicating that the Shp-2 activity is required for the full activation of the Akt pathway specifically if induced by the Ret^{M918T} mutant, are consistent with the indication that Ret^{M918T} may recruit specific molecules otherwise poorly recruited by the GDNF-stimulated Ret^{wt} or by Ret^{C634Y} (8, 31). Indeed, the threonine for methionine substitution at codon 918 lies within the catalytic domain of Ret and results in a shift in substrate specificity. As a consequence, the mutated Ret^{M918T} preferentially phosphorylates optimal sub-

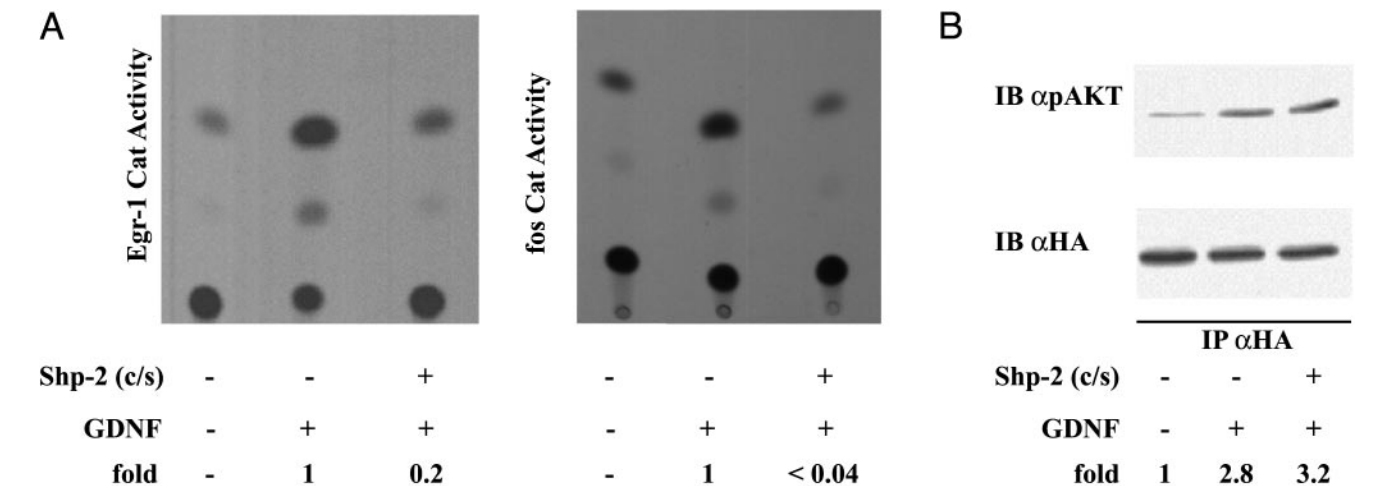


FIG. 5. Involvement of Shp-2 in GDNF-induced signaling. A, PC12- α 1/wt cells were transfected with pEgr-1-CAT (left panel) or pfos-CAT (right panel) together with Shp-2 (C/S), as indicated. 24 h later, GDNF (50 ng/ml) was added for 48 h, as indicated. The fold values of CAT activity in the presence of Shp-2 (C/S) mutant have been calculated over the control (in the absence of phosphatase) set to 1 (see also legend to Fig. 2). B, PC12- α 1/wt cells were transiently transfected with HA-Akt fusion protein together with Shp-2-C/S plasmid, as indicated. Thirty-six hours after the transfections, cells were starved for 12 h and then stimulated 5 min with GDNF (100 ng/ml). Cell lysates were immunoprecipitated (IP) with anti-HA antibodies, and immunoblotted (IB) with anti-pAkt (upper panel) or, to confirm equal loading, anti-HA (lower panel) antibodies.

strates for Src and Abl (8). On the other hand, a recent report (32) showing a stronger activation of Akt by the Ret^{M918T} mutant is consistent with our observation of the poor stimulation of this pathway by either GDNF-stimulated Ret^{wt} or by Ret^{C634Y}.

The implication of Shp-2 in Ret signaling looks in contrast with the evidence that, in NIH 3T3 cells, the overexpression of Shp-2 did not reduce the autophosphorylation of an active Ret mutant allele (11). Although here we show results obtained with different Ret mutants and cell system, the most plausible interpretation for this apparent discrepancy is that Ret likely neither bind Shp-2 nor is a direct substrate of the phosphatase activity. This interpretation is strongly supported by recent evidences showing that recruitment of Shp-2 to GDNF-stimulated Ret^{wt} is indirect and mediated by binding to a docking protein, even though the identity of the docking protein involved in Shp-2 binding is not unique and likely depends on the experimental cell system (9, 29). Furthermore, the results shown here were obtained either with GDNF-stimulated Ret^{wt} or with each of two different Ret mutants, thus indicating that in PC12 cells Shp-2 activity is crucial for Ret downstream signaling, though we cannot exclude that Shp-2 substrate(s) recruited to Ret mutants may be different (or present at different concentrations) in the different cell lines, *i.e.* in PC12 compared with NIH 3T3 cells. On the other hand, given that the related Shp-1 tyrosine phosphatase has opposite, or no, effects on Ret signaling (Ref. 11; and Califano, D., manuscript in preparation), it seems likely that Shp-2 acts in a specific way to mediate Ret signaling.

In summary, the experiments presented demonstrate that in PC12 cells both Ret^{M918T} and Ret^{C634Y} mutants may be found in complex with Shp-2, and indicate that this phosphatase mediates distinct functional interactions that depend on which Ret mutant was present in such a complex. These findings makes evident a main molecular difference between these Ret mutants and indicate this phosphatase as a key substrate involved in determining the difference between the MEN-2A and MEN-2B associated diseases.

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The Shp-1 and Shp-2, tyrosine phosphatases, are recruited on cell membrane in two distinct molecular complexes including Ret oncogenes

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Abstract

The Shp-2 and Shp-1 non-transmembrane tyrosine phosphatases display different and even opposing effects on downstream signaling events initiated by Ret activation. By using rat pheochromocytoma-derived PC12 cells, here we studied the interactions of Shp-2 and Shp-1 with two activated mutants of Ret receptor, Ret^{C634Y} and Ret^{M918T}. Each of these mutated receptors causes inheritance of distinct cancer syndromes, multiple endocrine neoplasia (MEN) type 2A and type 2B, respectively.

We show that: (i) both Shp-1 and Shp-2 are associated to a multiprotein complex that includes Ret mutants; (ii) the Shp-1-Ret complexes are distinct from Shp-2-Ret complexes, and these complexes are differently distributed inside and outside lipid rafts; (iii) constitutively activated Ret proteins neither directly bind to nor are substrates of these phosphatases. Our results well support the evidence that Ret complexes within and outside rafts mediate distinct biological functions, and indicate that the presence of either Shps participates to determine such functions.

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1. Introduction

The Ret protein is a transmembrane receptor belonging to the tyrosine kinase family. Germline mutations in the *ret* gene are responsible for the inheritance of neoplastic syndromes, multiple endocrine neoplasia (MEN) types 2A and 2B, and of familial medullary thyroid carcinoma. MEN2A and MEN2B are distinct hereditary neoplastic diseases, sharing several common features: i.e. development of medullary thyroid carcinomas and pheochromocytomas [1]. In the case of familial medullary thyroid carcinoma and MEN2A syndrome, the mutations most frequently found concern specific cysteines residues lo-

cated in the extracellular domain that are normally engaged in the formation of intramolecular disulphide bridge (Cys 609, 611, 618, 620, and 634). These mutations result in the formation of an unpaired cysteine residue, leading to constitutive dimerisation by intermolecular disulphide bridge of two mutated Ret molecules. Conversely, a single point mutation, which results in a Thr for Met substitution at codon 918, within the Ret catalytic domain, is the most frequent mutation in MEN2B syndrome, that involves an intramolecular mechanism to convert Ret into a dominant transforming gene. Both MEN2A and MEN2B associated mutations cause constitutive activation of the Ret tyrosine kinase activity and lead to the formation of cancers in neuroendocrine organs [2–4].

Four distinct ligands, for the wild-type c-Ret protein, have been identified, all of which are related to the glial cell line-derived neurotrophic factor (GDNF). Stimulation of c-Ret activity implicates the recruitment of the receptor

Abbreviations: GDNF, glial cell line-derived neurotrophic factor; GST, glutathione S-transferase; MEN, multiple endocrine neoplasia; Shp-1, SH2-containing tyrosine phosphatase 1; Shp-2, SH2-containing tyrosine phosphatase 2.

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protein to a multi-component complex that, besides the ligand, includes one of the four members of the GPI-anchored protein GFR α family, GFR α 1–4, leading to Ret dimerisation and autophosphorylation at specific cytoplasmic tyrosine residues [5,6].

Shp-2 and Shp-1 are non-transmembrane phosphotyrosine phosphatases involved in protein tyrosine kinase signal transduction events [7]. These two SH2-containing phosphatases, although structurally related, have non-redundant and even opposed signaling roles. Shp-1 is predominantly expressed in cells of hematopoietic origin, and likely serves as negative regulator of most hematopoietic-specific signaling systems. On the other hand, Shp-2 is ubiquitous and functions as an important signal transducer for several receptor tyrosine kinases and cytokine receptors [7–13].

Compelling evidence from several laboratories indicate that both Shp-2 and Shp-1 are implicated as downstream mediators of Ret signalling, initiated both by GDNF-stimulation of *wild type* Ret and by the active Ret mutant proteins [14–17]. However, the function of each of these phosphatases and the molecular partners involved are still largely unknown. As recently reported the GDNF-stimulated c-Ret transiently associates with membrane cholesterol rich microdomains, named lipid rafts [18,19]. Here we investigated whether constitutively active Ret mutants associate with rafts and whether these mutants bind Shp-2 and Shp-1 outside or inside the lipid rafts. To this aim we studied two distinct mutants, Ret^{C634Y} and Ret^{M918T}, causing the MEN2A and MEN2B syndromes, respectively. In Ret^{C634Y}, the mutation is localised in extracellular domain and causes constitutive dimerization of the receptor molecule, while Ret^{M918T}, is mutated in the catalytic domain and thus activated by an intramolecular mechanism. Furthermore, because of the different modalities of activation of Ret^{C634Y} and Ret^{M918T}, we compared the interactions of these phosphatases with each of the two Ret mutants.

We found that, at steady state, a quote of both Ret mutant proteins is present in lipid rafts, in the rat pheochromocytoma-derived cell line, PC12. However, the complexes constituted by Shp-2 bound to either Ret^{C634Y} or Ret^{M918T} are mainly localised outside raft. As the activity of the Shp-2 phosphatase is needed to transmit Ret signaling to the nucleus [17], our data indicate that important steps of the signaling events, initiated by these oncogenes, occur outside the raft compartment.

2. Materials and methods

2.1. Cell culture and stimulations

PC12 cells were grown in RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine, 10% horse serum and 5% fetal calf serum. PC12/wt, PC12/Ret^{C634Y} and PC12/Ret^{M918T}, which are PC12-derived cell lines expressing the human *RET9* isoform of either the wild type (Ret^{wt}),

or with the C634Y mutation (Ret^{C634Y}) and with the M918T mutation (Ret^{M918T}), respectively, were generated and grown as previously reported [20]. PC12- α 1/wt cells, expressing both Ret^{wt} and the GFR α 1 protein, were obtained and grown as reported [21]. When indicated, GDNF (Promega) has been added to the culture medium.

2.2. Constructs

A glutathione *S*-transferase fusion protein encoding amino acids 1–251 of Shp-1 was generated by polymerase chain reaction from the full-length Shp-1 cDNA using the primers 5' -ATGAGATCTATGCTGTCCCGTGGGTGG-3' and 5' -CATGAATTCTTACTCAAACCTCCAGAA-3'. The resulting PCR product was digested with *Eco*RI and *Bgl*II and ligated into pGEX-4T3 to yield GST-(N+C)-Shp1. GST-(N+C)-Shp1 protein was produced from *Escherichia coli* and purified on glutathione sepharose affinity chromatography. The GST-(N+C)-Shp2 plasmid was kindly provided by B. Neel [22].

2.3. Antibodies, immunoprecipitations and immunoblotting

Cells were washed twice in ice-cold PBS, and lysed in buffer A (50 mM Tris–HCl pH 8.0 buffer containing 150 mM NaCl, 1% Nonidet P-40, 2 μ g/ml aprotinin, 1 μ g/ml pepstatin, 2 μ g/ml leupeptin, 1 mM Na₃VO₄). Protein concentration in the cell extract was determined by the Bradford assay using bovine serum albumin as the standard. For immunoprecipitation, cell extracts (1 mg) were incubated with specific antibodies for 2 h at 4 °C and then immunoprecipitated with protein G-plus agarose (Calbiochem, Oncogene Research) overnight at 4 °C. Immunoprecipitates were washed five times with the above buffer A and denaturated in Laemmli buffer for 5 min at 100 °C, then subjected to SDS-PAGE (8% acrylamide). Gels were electroblotted into polyvinylidene difluoride membranes (Millipore, Bedford, MA), and detected with the indicated primary antibodies and peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system (Amersham-Pharmacia Biosciences). Primary antibodies used were: anti-Ret (H-300), anti-Shp-1 (C-19) and anti-Shp-2 (C-18) (Santa Cruz Biotechnology, CA), anti-phosphotyrosine monoclonal antibodies (4G10) from Upstate Biotechnology (Lake Placid, NY), anti-GST (Amersham-Pharmacia Biosciences). When indicated, membranes were stripped in 62.5 mM Tris–HCl pH 6.7, 0.1 M 2-mercaptoethanol, 2% SDS, for 30 min at 55 °C. For immunoblots shown in the figures, statistical analysis was performed on at least four independent experiments.

2.4. Sucrose gradients

Cellular lysates derived from PC12- α 1/wt, PC12/Ret^{C634Y} and PC12/Ret^{M918T} cell lines were analysed by sucrose gradient centrifugation according to previously

published protocols [23,24]. Cells were washed twice in ice-cold PBS, then lysed in buffer B (20 mM Tris–HCl pH 8.0 containing 15 mM NaCl, 1% Triton X-100, 10 mM EDTA, 2 µg/ml aprotinin, 1 µg/ml pepstatin, 2 µg/ml leupeptin, 1 mM Na₃VO₄). Lysates volume were brought to 40% sucrose, and placed at the bottom of a centrifuge tubes. A sucrose step gradient (5–35% in 20 mM Tris–HCl pH 7.5, 15 mM NaCl and 10 mM EDTA) was layered on top, and the samples were centrifuged at 260000 × g for 18–20 h in a SW41 rotor Beckman (Fullerton, CA). One-milliliter fractions were harvested from the top of the gradient. Collected fractions were trichloroacetic acid (20% TCA) precipitated, solubilized in Laemmli buffer, boiled for 5 min before running on 8% SDS-PAGE and immunoblotted with specific antibodies. Where indicated, immunoprecipitation of distinct proteins was performed on the different sucrose gradient fractions.

2.5. Far Western blotting, GST pull-down assay

GST-fusion proteins, GST-(N+C)-Shp1, GST-(N+C)-Shp2 containing both SH2 domains of Shp-1 or Shp-2, and GST-Shc/FL were expressed in *E. coli*, purified on glutathione-Sepharose beads [22] and eluted by incubating with 20 mM glutathione in 150 mM NaCl, 50 mM Tris–HCl pH 7.4. In Far Western blotting experiments, 200 µg of total cell lysates was subjected to SDS-PAGE (8% acrylamide). Proteins were electroblotted to polyvinylidene difluoride membranes. Filters were blocked with 5% non-fat dry milk in Tris-buffered saline/Tween, incubated with GST-fusion proteins 1.5 µg/ml, probed with monoclonal GST antibodies. In GST Pull-down assay, 1 mg of total lysates was incubated for 1 h at 4 °C with 30 µl of glutathione-Sepharose (Amersham Pharmacia Biotech) and 50 µg of GST-fusion protein. The beads were washed three times with buffer B, than 30 µl of 2 × Laemmli buffer were added and the mixture was boiled for 5 min. Samples were subjected to SDS-PAGE (8% acrylamide). Proteins were immunoblotted using anti-Ret (H300) antibodies.

2.6. Phosphatase assay

One milligram of total cell extract was immunoprecipitated for 2 h at 4 °C with either anti-Shp1 or anti-Shp2 antibodies in the presence of protein G-plus agarose. The beads were washed two times with buffer B and two times with buffer C (5 mM EDTA, 50 mM HEPES and 10 mM DTT). The reaction was started by adding 40 µl of reaction buffer [buffer C plus 20 nM microcystin–leucine–arginine (Alamone Laboratories, Jerusalem, Israel) and 10 µM ZnCl₂] and carried out at 30 °C up to 30 min. Reactions were stopped by adding 20 µl of 3 × Laemmli buffer. After denaturation samples were subject to 8% SDS-PAGE, proteins were immunoblotted using anti-pTyr antibodies.

3. Results and discussion

3.1. Shp-1 and Shp-2 participates to distinct multiprotein complexes that include Ret

Because both phosphatases have been described to modulate Ret oncogene intracellular signaling, we analysed the molecular complexes constituted by Ret active proteins together with Shp-2 and/or Shp-1 phosphatases, and asked whether each phosphatase is recruited in the same or in distinct protein complexes. To this aim, we took advantage of PC12-derived cell lines, in which either Ret^{wt} or the mutated alleles Ret^{C634Y} and Ret^{M918T} (PC12/wt, PC12/Ret^{C634Y}, and PC12/Ret^{M918T} cells, respectively) are constitutively expressed. The cell lysates were immunoprecipitated with either anti-Shp-1 (Fig. 1A, left side), or anti-Shp-2 (right side) antibodies and immunoblotted with anti-pTyr (panel a), anti-Ret (panel b), anti-Shp-1 (panel d) and anti-Shp-2 (panel c) antibodies. As shown, the constitutively active forms of Ret (Ret^{C634Y} in PC12/Ret^{C634Y} and Ret^{M918T} in PC12/Ret^{M918T}), but not the unstimulated Ret^{wt} (in PC12/wt cells) were found in the same immunocomplex with each phosphatase (Panels a and b, lanes 3, 4, 7 and 8). On the other hand, Shp-1 immunoprecipitates did not contain Shp-2 (Panel c, lanes 1 to 4) and, conversely, Shp-2 immunoprecipitates did not contain Shp-1 (Panel d, lanes 5 to 8), thus suggesting that the two complexes are distinct and each phosphatase may bind to the activated Ret mutants but not to the cognate phosphatase. Therefore, to confirm that the two phosphatases are not in the same complex, we performed two further immunoprecipitation rounds. First, in order to deplete the supernatants, resulting from the above immunoprecipitations, of the residual Shp-1- and Shp-2-bound Ret molecules, we immunoprecipitated each supernatant again with the same antibody (Fig. 1B). Subsequently, the supernatants derived from the second immunoprecipitations with anti-Shp-1 antibodies were immunoprecipitated with anti-Shp2 antibodies (Fig. 1C, left side), and the supernatants from the second immunoprecipitations with anti-Shp-2 antibodies were immunoprecipitated with anti-Shp-1 antibodies (right side). Shp-2-bound Ret was now clearly found in supernatants from Shp-1 immunoprecipitates (upper panel, lanes 3 and 4) and conversely Shp-1-bound Ret was found in supernatants from Shp-2 immunoprecipitates (upper panel, lanes 7 and 8). Taken together, these results indicate that two distinct molecular complexes may take place, one including Ret-Shp-1 and the other Ret-Shp-2 molecules. These interactions likely need the activation of Ret as the unstimulated Ret^{wt} was unable to form complexes with neither phosphatase.

3.2. Distribution of Ret and SH2-containing tyrosine phosphatases in and outside lipid rafts

An important aspect of GDNF signaling through Ret resides in its ability to cause the stimulated receptor to compartmentalize in the membrane cholesterol rich micro-

domains, named lipid rafts [19,25]. In turn, GDNF-induced delocalization of Ret^{wt} to rafts causes a shift in substrate recruitment to the receptor [25]. Therefore, given that both Ret oncogenic mutants interact with either Shp-1 or Shp-2 phosphatases, although forming distinct complexes, we

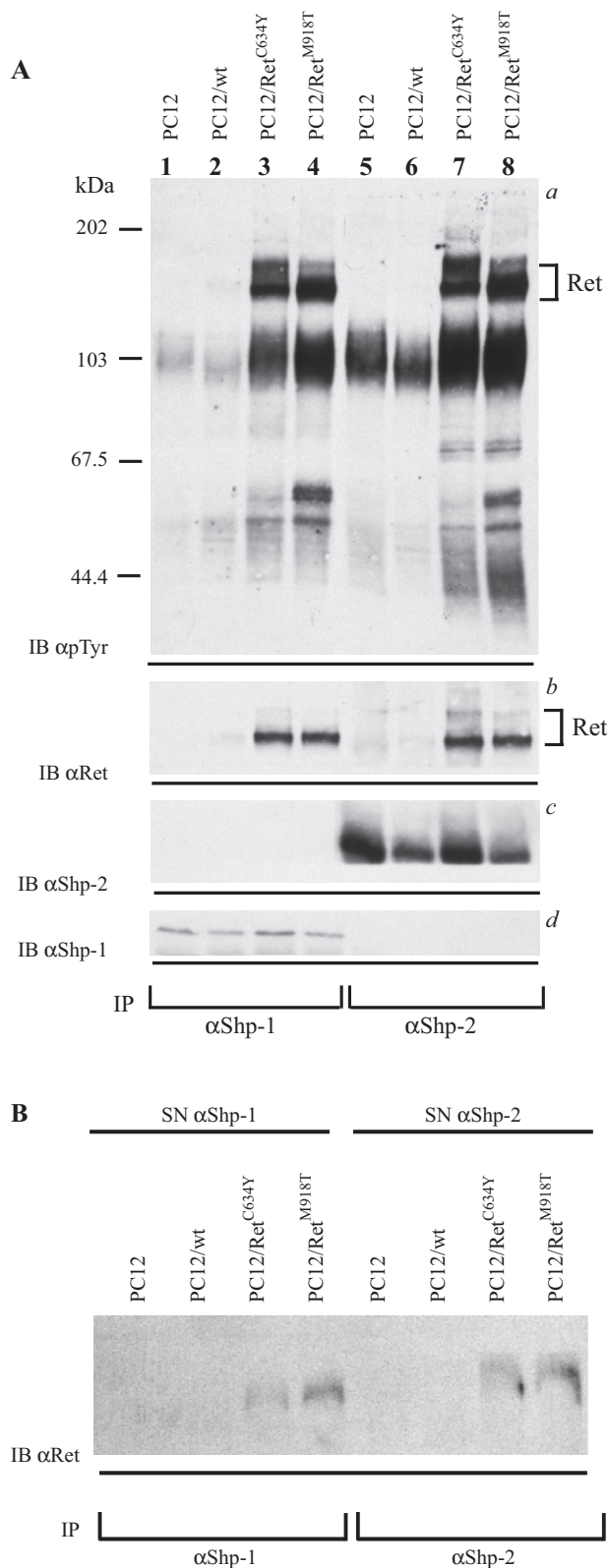


Fig. 1. (A) Cell lysates of the indicated cell lines were immunoprecipitated (IP) with anti-Shp-1 (left) and anti-Shp-2 (right) antibodies and immunoblotted (IB) with anti-pTyr (a), filter was stripped, cut and blotted with anti-Ret (b), anti-Shp-2 (c) and anti-Shp-1 (d) antibodies. (B) The supernatants from Shp-1 and Shp-2 immunoprecipitates were again immunoprecipitated each with the same antibody (as indicated) and blotted with anti-Ret antibody. (C) The supernatants obtained from the second immunoprecipitations with anti-Shp-1 antibodies were immunoprecipitated with anti-Shp2 antibodies (left) and supernatants obtained after the second immunoprecipitations with anti-Shp-2 antibodies were immunoprecipitated with anti-Shp-1 antibodies (right) and immunoblotted with anti-Ret, anti-Shp2 and anti Shp1 antibodies, as indicated.

asked whether such complexes localize in distinct plasma membrane microdomains. By using floating sucrose gradients, we first analysed the partitioning in membrane rafts of Shp-1, Shp-2 and Ret active mutants (Ret^{C634Y} and Ret^{M918T}). As shown in Fig. 2A, significant amounts of both Ret mutants, 7.6% of Ret^{C634Y} and 8.0% of Ret^{M918T}, but not Ret^{wt}, were found associated with the detergent-insoluble lipid raft-containing fractions (Fig. 2C and compare Fig. 2A panels a to panel a in Fig. 2B). Furthermore, the analysis of distribution of the tyrosine phosphorylated Ret molecules indicates that in the case of Ret^{M918T} monomers, that are activated by an intramolecular mechanism, the enrichment of tyrosine phosphorylated molecules in the floating fractions was less drastic than in the case of tyrosine phosphorylated Ret^{C634Y}, that spontaneously form stable homodimers (Fig. 2A compare panel left-d to right-d). Such distributions suggest that the formation of active homodimers would contribute to the accumulation, or stability, of Ret within the raft fractions. Furthermore, we analysed the distribution of the Shp-1 or Shp-2 phosphatases. In PC12-α1/wt cells, in which the Ret^{wt} is inactive, around 20% of Shp-1 is found in the rafts fractions (Fig. 2B panel b and Fig. 2D). In contrast, in both PC12/Ret^{C634Y} and PC12/Ret^{M918T} cells, the presence of Shp-1 within the raft frac-

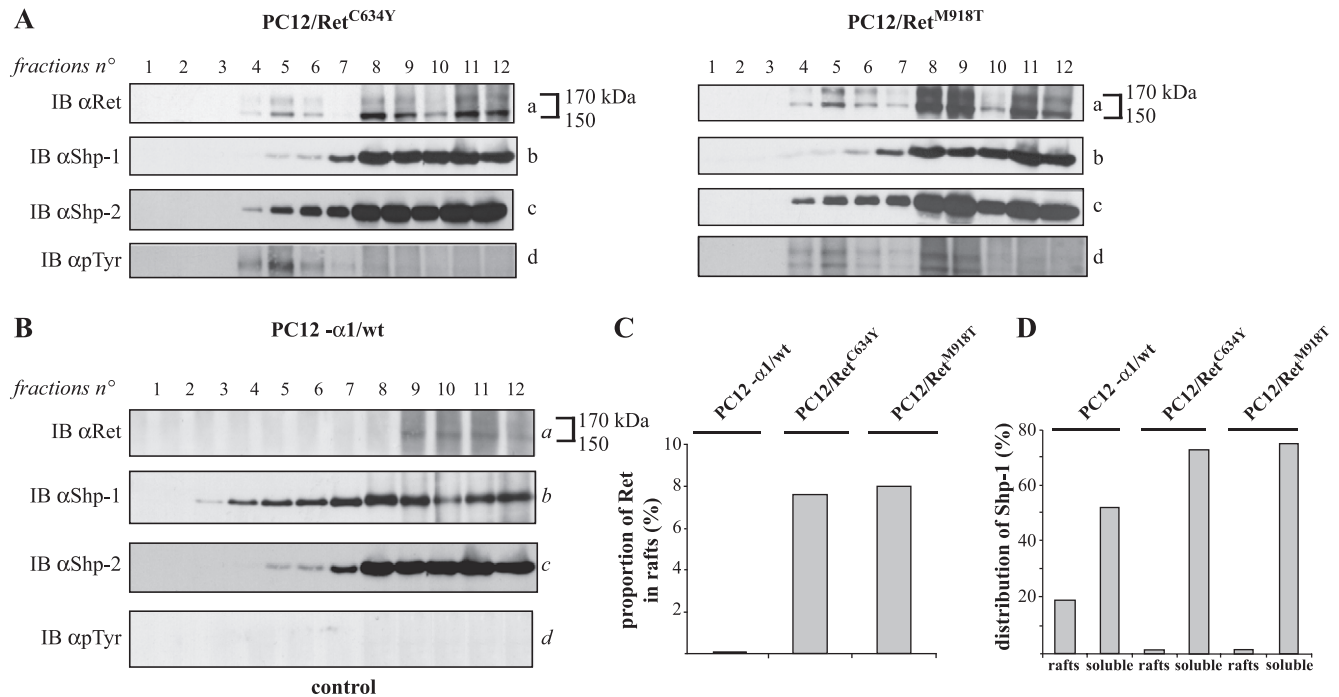


Fig. 2. (A) Sucrose gradient fractions of Triton X-100 lysates prepared from PC12/Ret^{C634Y} (left panel) and PC12/Ret^{M918T} (right panel) cell lines were analysed for the presence of Ret (a), Shp-1 (b), Shp-2 (c) and pTyr (d) proteins using specific antibodies, as indicated. Rafts proteins are in fractions 4 to 6, while detergent-soluble proteins are in fractions 9 to 12. (B) Sucrose gradient fractions of Triton X-100 lysates prepared from unstimulated PC12α-1/wt cell line were analysed as described above. (C) The amount of Ret molecules in each gradient fraction has been determined using the NIH-image program. The histogram reports the relative amount of Ret^{wt}, Ret^{C634Y} and Ret^{M918T} inside lipid rafts, as percent of total Ret molecules. (D) For each cell lines, the histogram reports the amount of Shp-1 inside and outside lipid rafts, as percent of total Shp-1 molecules. Contamination in the floating part of the gradient has been excluded by hybridising all fractions with anti transferrin receptor antibodies (not shown).

tions is difficult to appreciate (Fig. 2A panels b and Fig. 2D). This distribution is quite specific for Shp-1, since the cognate Shp-2 phosphatase is more homogeneously distributed all along the gradient and with few differences between the control PC12-α1/wt and the PC12/Ret^{C634Y} or the PC12/Ret^{M918T} cells (compare Fig. 2B panel c to Fig. 2A, panels left-c and right-c). Distribution of phosphatases indicates that the presence of active Ret molecules (either Ret^{C634Y} or Ret^{M918T}) may induce the exclusion of Shp-1, but not of Shp-2 from the raft compartment.

Taken together these results show that: (1) in analogy with the GDNF-stimulated Ret^{wt}, a portion of the active Ret^{C634Y} and Ret^{M918T} mutant proteins are localised in lipid rafts; (2) expressing either Ret^{C634Y} or Ret^{M918T} mutants causes enrichment of Shp-1 in Triton X-100-soluble fractions as compared to PC12-α1/wt cells, that express the inactive Ret^{wt} receptor.

3.3. Shp1 and Shp2-Ret complexes are localized in different membrane domains

Given that active Ret proteins are enriched within the raft fractions, it seems plausible that either the Shp-1 or the Shp-2-containing Ret complexes might be preferentially localised in these fractions. Therefore, we next determined their distribution in Ret-transfected PC12 cells. To this

aim, we used either anti-Shp-1 or anti-Shp-2 antibodies to immunoprecipitate the sucrose gradient fractions corresponding to rafts, and to Triton X-100-soluble proteins and analysed by immunoblot with anti-Ret antibodies. As shown, Shp-1 binds, either directly or indirectly, both Ret^{C634Y} (Fig. 3A, left panel) and Ret^{M918T} (Fig. 3B, left panel). Co-immunoprecipitates were clearly found both in rafts and in the soluble fractions. Therefore, in order to compare the relative amounts of Shp-1-bound Ret molecules in each fraction, in Fig. 3A and B (left panels), we show serial dilutions of the samples from the Triton X-100-soluble fractions (9–12), in which the Shp-1 signal was saturated. Despite the higher absolute amounts of both Ret and Shp-1 outside rafts, when comparing the relative amounts of Shp-1-bound Ret (either Ret^{C634Y} or Ret^{M918T}) in fraction 6 to dilution 1/50 of fraction 9–12, binding of Shp-1 to Ret is strongly enhanced in the rafts fractions. Furthermore, we compared PC12/Ret^{C634Y} to PC12/Ret^{M918T} for partitioning of Ret-Shp-1 complexes within rafts to complexes present outside rafts (Fig. 3C). As shown, the relative amounts of Shp-1-bound Ret^{C634Y} found in rafts (approximately 69.0% of total Shp-1-bound Ret^{C634Y}) were clearly higher than those of Shp-1-bound Ret^{M918T} in rafts (being less than 13.7% of total Shp-1-bound Ret^{M918T}). Whether the preferential enrichment of Shp-1-bound Ret^{C634Y} active proteins in rafts would de-

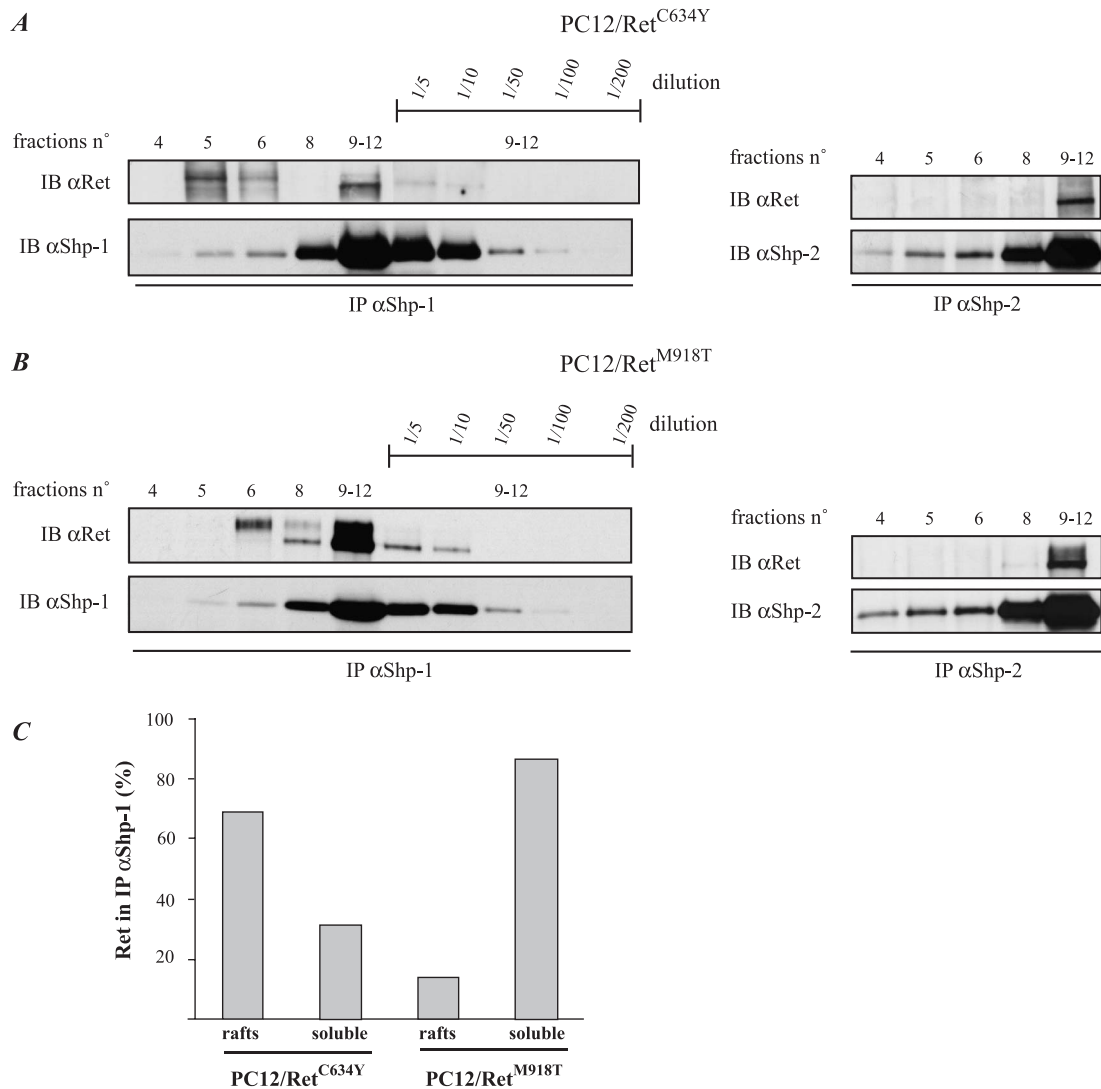


Fig. 3. Cell lysates from PC12/Ret^{C634Y} (A) and PC12/Ret^{M918T} (B) cell lines were fractionated on sucrose gradient as described in Fig. 2. Individual fractions corresponding to lipid rafts (4, 5, 6), fraction 8 and a pull of fractions 9 to 12 (membrane-soluble proteins) were immunoprecipitated (IP) with anti-Shp-1 (left panels) or anti-Shp-2 (right panels) antibodies, and analysed with anti-Ret, anti-Shp-1 and anti-Shp-2 antibodies, as indicated. When indicated, sequential dilutions (5 to 200 times) of protein lysates from the pull of fractions 9 to 12 were analysed as described above. (C) The histogram shows the relative amount of Shp-1-bound Ret molecules present in rafts fractions and in soluble fractions, from the indicated cell lines, as percent of total Shp-1 molecules.

pend on a receptor specific conformation determined by the different mechanisms of activation of each Ret mutant still remain to be ascertained. If this was the case, a possible explanation may reside on the assumption that receptor homodimerization would stabilize the permanence of Ret within the rafts. This possibility is further supported by the different partitioning of phosphorylated Ret mutant proteins, Ret^{C634Y} accumulating within the rafts and Ret^{M918T} being found all along the gradient (Fig. 2A, right panel).

On the other hand, when using anti-Shp-2 antibodies, both Ret^{C634Y} and Ret^{M918T} mutated receptors are co-immunoprecipitated together with Shp-2 exclusively within the Triton X-100-soluble fractions (Fig. 3A and B, right panels, fractions 9 to 12 pulled). Taken together these results well support the observation (see Fig. 1) that the complexes

formed by Ret with Shp-1 or with Shp-2 are distinct and likely correspond to distinct functional states of the active receptors.

3.4. In PC12/Ret^{C634Y} and PC12/Ret^{M918T} cells the Shp-1 partners are distinct from those of Shp-2

Next we addressed the direct protein partners of Shp-1 and Shp-2 in PC12/Ret^{C634Y} and PC12/Ret^{M918T} cells. To this purpose, GST-fusion proteins containing the SH2 domains of either Shp-1 (Fig. 4A, left panel), or Shp-2 (middle panel) were used to probe blots of total cell lysates from PC12/Ret^{C634Y} and PC12/Ret^{M918T} cell lines. As several cellular proteins are phosphorylated in tyrosine also by growth factors present in the serum, in order to exclude binding of the GST-fusion proteins to unspecific targets, we

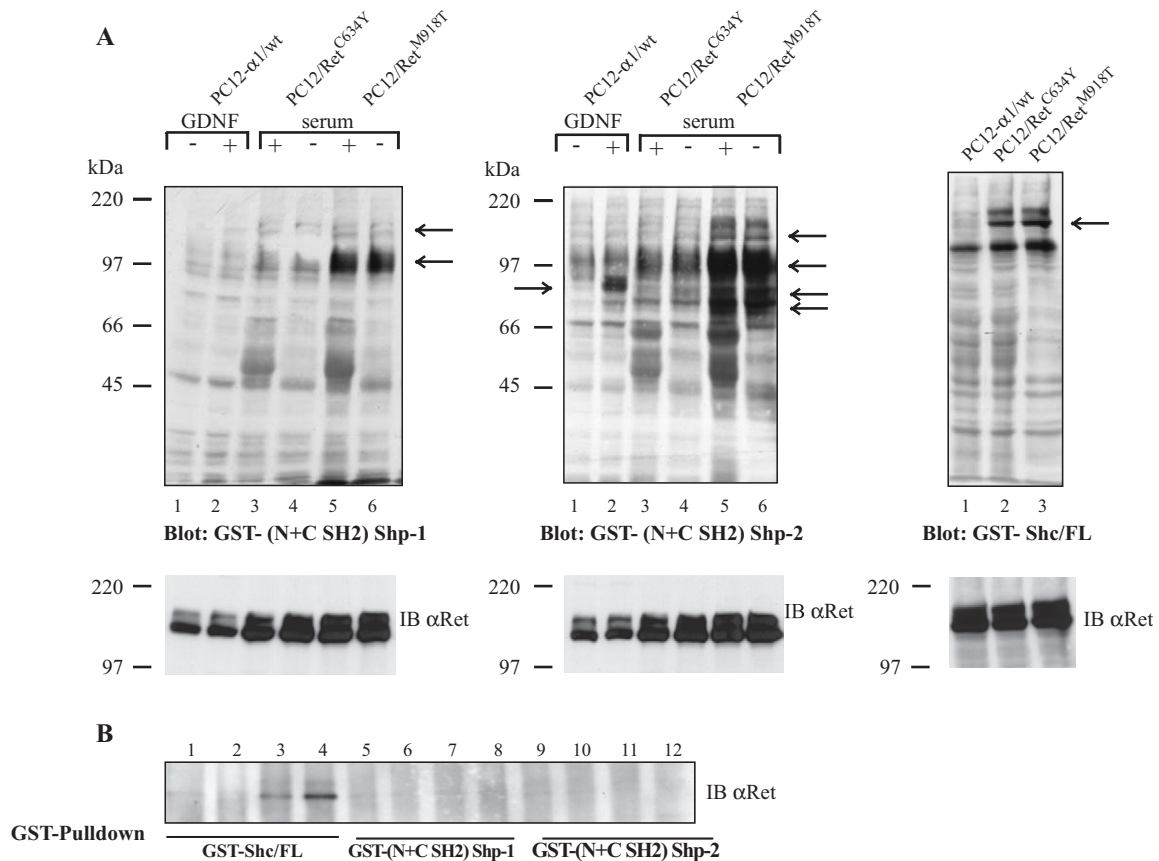


Fig. 4. (A, upper panels) Far-Western blot analysis of cell lysates from the indicated cell lines using GST- (N+C SH2) Shp-1 (left panel), GST-(N+C SH2) Shp-2 (middle panel) and GST-Shc/FL (right panel) fusion proteins. Lanes 1 and 2: PC12- α 1/wt cell line either left unstimulated (–) or stimulated with GDNF (+); lanes 3 and 4: PC12/Ret^{C634Y} cell lines growth in presence (+) or in absence (–) of serum; lanes 5 and 6: PC12/Ret^{M918T} cell lines growth in presence (+) or in absence (–) of serum. (A, lower panels) filters were stripped and reprobed with α Ret antibody. (B) GST Pull-down experiments using GST-Shc/FL (lanes 1 to 4), GST-(N+C SH2) Shp-1 (lanes 5 to 8) and GST-(N+C SH2) Shp-2 (lanes 9 to 12) fusion proteins. Lanes 1, 5 and 9: PC12- α 1/wt cell line unstimulated; lanes 2, 6 and 10: PC12- α 1/wt cell line GDNF-stimulated; lanes 3, 7 and 11: PC12/Ret^{C634Y} cell lines; lanes 4, 8 and 12: PC12/Ret^{M918T} cell lines.

included in the same blot cell lysates from PC12/Ret^{C634Y} and PC12/Ret^{M918T} cells grown in absence of serum (lanes 4 and 6 in each panel). Moreover, we compared binding to phospho-tyrosine containing proteins following acute stimulation of Ret^{wt}. To this aim, we included in the same blot, cell lysate from PC12- α 1/wt cells (in which are expressed both the Ret^{wt} and the GFR α -1) harvested either upon 10 min of GDNF stimulation or left untreated (lanes 2 and 1, respectively, in each panel). As shown in the left panel, using the GST-SH2/Shp-1 fusion protein as probe, in PC12/Ret^{M918T} and, at much lesser extent, in PC12/Ret^{C634Y} a band of 97-kDa apparent MW was clearly detected (compare lane 1 to 4 and 6). This band was absent in the PC12- α 1/wt cells both if stimulated with GDNF and if left untreated. The presence of a faint double band compatible with Ret size was also detected in PC12/Ret^{C634Y} and PC12/Ret^{M918T} and in the stimulated PC12- α 1/wt (compare lane 1 to lanes 2, 3 4, and 6, respectively, see arrows).

Further, we performed parallel experiments, using the GST-SH2/Shp-2 fusion protein to overlay the blot, and detected several specific bands in PC12/Ret^{C634Y}, in

PC12/Ret^{M918T} and in the GDNF-stimulated PC12- α 1/wt cell lysates (Fig. 4A, middle panel). These include two bands of approximately 80 and 75 kDa that were common to the three cell lines (compare lanes 2, 4 and 6 to lane 1), and an additional band of approximately 97 kDa present in PC12/Ret^{M918T} and, at lower extents, in PC12/Ret^{C634Y} (compare lane 2 to lanes 4 and 6). The 97-kDa band can be identified with Gab2 [17, and data not shown], while the identity of the other bands remains to be determined. In PC12/Ret^{M918T}, but in no other tested cell lysates, two bands of around 170 kDa, whereas well recognized by the GST-SH2/Shp-2 protein (compare lanes 6 to 1), and migrate with a size that is compatible with Ret (Fig. 4A, lower panels). Overlay with the GST-Shc/FL protein was performed to confirm the specificity of the overlay assay (right panel).

The results indicate that Ret active proteins induce both Shp-1 and Shp-2 to interact via their SH2 domains with several cellular proteins. Indeed, Shp-1 directly binds one or few proteins that are present in PC12/Ret^{M918T} and, even if at lower extents, also in PC12/Ret^{C634Y} and in GDNF-stimulated PC12- α 1/wt cell lysates. On the other hand,

Shp-2 seems to interact with a more complex array of partners, likely including Gab2.

Given that GST-SH2/Shp-2 binds to Ret in PC12/Ret^{M918T} cells and GST-SH2/Shp-1, though almost undetectable, binds Ret in both PC12/Ret^{C634Y} and PC12/Ret^{M918T}, we performed pull-down assays of Ret proteins with GST-SH2/Shp-1 and GST-SH2/Shp-2 and compared to pull-down with the GST-Shc/FL protein, as control. As shown in Fig. 4B, the GST-SH2/Shp-1 (lanes 5–8) and GST-SH2/Shp-2 (lanes 9–12) proteins were unable to bind neither the GDNF-stimulated Ret^{wt} nor the active both Ret^{C634Y} and Ret^{M918T} mutated proteins. These results confirm that both phosphatases are recruited to Ret by binding to cellular intermediaries.

3.5. Ret proteins are not substrates of Shps

Further, to determine whether the tyrosine phosphorylated Ret mutants are substrates for the Shp-1 or Shp-2 phosphatases, we performed an in vitro phosphatase activity assay. Either Shp-1- or Shp-2-bound complexes were immunoprecipitated from each lysate (from PC12/Ret^{C634Y} and

PC12/Ret^{M918T} cells), and incubated in the phosphatase buffer for increasing times (Fig. 5A and B, respectively). Each complex was then subjected to immunoblot analysis with anti-phosphotyrosine antibody. As shown (Fig. 5A), in the Shp-1-bound complex, the levels of tyrosine phosphorylated Ret (either Ret^{C634Y} or Ret^{M918T}) remained constant up to 30 min of incubation. As internal control, in the same blot, is shown an unidentified tyrosine phosphorylated band, migrating as a 97 kDa that rapidly decreased within the first few minutes of incubation (see arrow). Similarly, in the Shp-2-bound complex (Fig. 5B), the levels of tyrosine phosphorylated Ret^{C634Y} and Ret^{M918T} remained constant up to 10 min of incubation. As control a tyrosine phosphorylated band of approximately 80–85 kDa was rapidly dephosphorylated within the first few minutes (see arrow). A slightly decreased dephosphorylation of Ret^{C634Y} for longer times of incubation likely reflects a sub-optimal use of Ret as substrate. Taken together (Figs. 4 and 5), these results indicate that tyrosine phosphorylated Ret mutants neither directly bind nor are good substrates for Shp-1 or Shp-2 tyrosine phosphatases. Although this result is in apparent contrast with the conclusions of a recent report, these

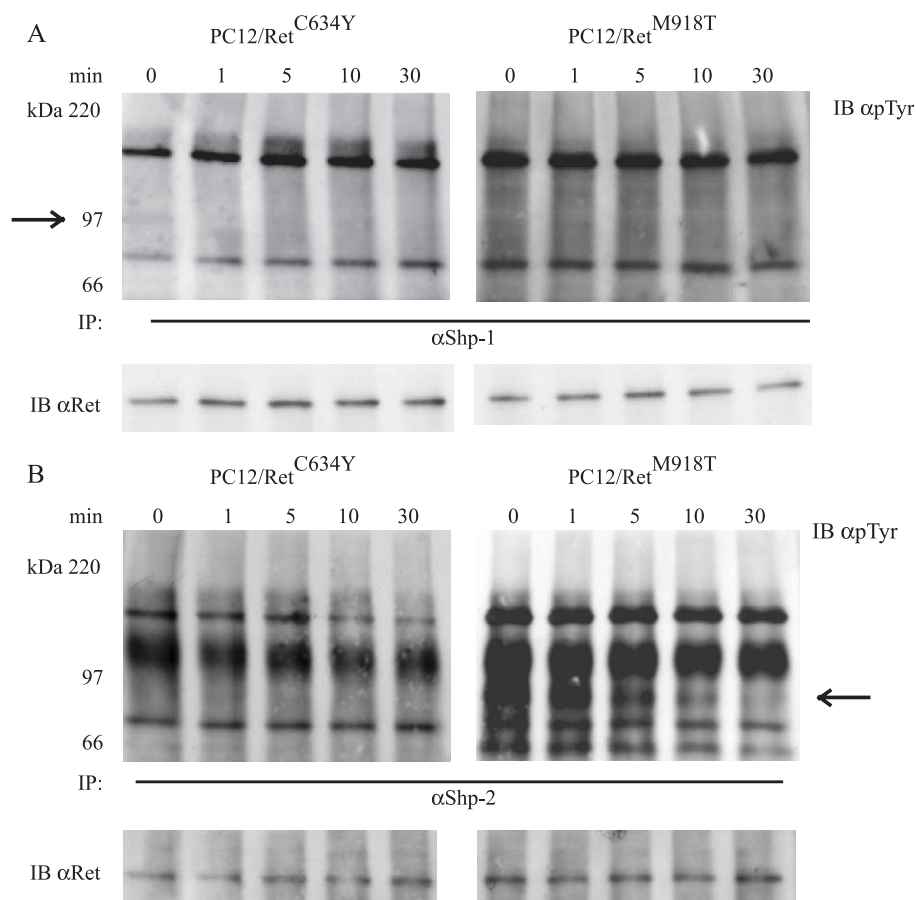


Fig. 5. In vitro dephosphorylation of Shp-1 or Shp-2-associated proteins. Cell extracts of PC12/Ret^{C634Y} and PC12/Ret^{M918T} cell lines were immunoprecipitated (IP) by either anti Shp-1 (A) or Shp-2 (B) antibodies. The precipitates were washed twice with lyses buffer without phosphatase inhibitor and twice with phosphatase buffer and then incubated in 40 μ l of reaction buffer for 0, 1, 5, 10 and 30 min at 30 °C. The reaction was analysed using anti-PY antibody. (A and B, lower panels) Filters were immunoblotted (IB) with α Ret antibody to confirm the equal amount of Ret molecules in each lanes.

differences may likely rely on the different approach used to determine binding and activity of Shp-1 on Ret mutants, that was mainly based on stable transfectants over-expressing the phosphatase and monitored by indirect assays [16].

3.6. Concluding Remarks

In epithelial and neuronal cells, Shp-2 is abundant and mediates signal transmission through several receptor tyrosine kinases, including Ret [17]. On the other hand, in such cells, where it is poorly expressed, Shp-1 has rather inhibitory effects on intracellular signaling from membrane receptors [26]. Here we show that Shp-2 and Shp-1 may both form complexes each including Ret active mutants but these complexes are distinct. Though this was not surprising because of the opposing effects of these phosphatases on Ret downstream signaling, the use of constitutive active mutants of Ret enabled us to demonstrate that each phosphatase acts preferentially in a distinct membrane compartment. A rather specific aspect of GDNF signaling through Ret is determined by the induction of the receptor component to move within raft microdomains upon ligand stimulation. In turn, localisation within the rafts enables Ret to recruit and activate effectors that are different from those recruited outside rafts [25]. Two mechanisms have been evoked for the localization of stimulated Ret^{wt} in rafts: the first implicates physical interaction of the extracellular domain with the GPI anchored GFR α 1 and the second needs interactions of the tyrosine-phosphorylated intracellular domain of the receptor with substrates resident in rafts, as for example FRS2/Snt. In analogy with what described for the stimulated Ret^{wt}, here we present evidence that a quote of both the mutated Ret^{C634Y} and Ret^{M918T} is as well localised in the raft fractions. For what it concerns the tyrosine phosphorylated Ret^{M918T}, rafts were not enriched in tyrosine-phosphorylated receptors as compared to the higher density gradient fractions. In contrast, in the case of Ret^{C634Y}, the portion of molecules localised in rafts, though it is a minor portion, is highly enriched in tyrosine-phosphorylated receptors (Fig. 2A, panels h and d). Whether homo-dimerization of Ret^{C634Y} stabilises its localization to the rafts compartment remains to be determined. Given that both Ret mutants are activated in absence of extracellular binding to neither GFR α -1 nor GDNF, it is conceivable to hypothesize that clustering in rafts is mediated by interacting with intracellular substrates [C. Ibanez, comment in Trends in Neurosciences (2001) 24; p. 429]. The presence in rafts of the mutated but not of wild-type receptors well supports the evidence of a non-GFR mediated mechanism that causes the clustering of tyrosine-phosphorylated Ret oncoproteins in these fractions.

3.6.1. Is specific signaling the only need for Ret to move in rafts?

In analogy with Ret^{wt}, it seems likely that mutant Ret proteins are in a dynamic equilibrium between non-raft and

raft compartments, where they may interact with different substrates (some of which specifically enriched in rafts). As stated above, in the case of both Ret^{C634Y} and Ret^{M918T}, the pool of Shp-1-bound Ret is distinct from the Shp-2-bound Ret. Indeed (cf. Fig. 3) the first complex takes place mainly in rafts while the Shp-2-bound complex is only evident outside rafts. On the other hand, as we recently reported [17], Shp-2 activity is required for Ret signaling through Ras and, at least in the case of Ret^{M918T}, through Akt. Therefore, because of the Shp-2 involvement in Ret signaling, it seems reasonable to assume that a crucial contribution to mutant Ret signaling takes place outside rafts.

On the other hand, the specific involvement of Shp-1 in rafts remains to be investigated. Though in both cell lines, the portion of the general pool of Shp-1 molecules present in rafts is poor (Fig. 2), the portion of Shp-1 molecules bound in complex to Ret is enriched in rafts microdomains (Fig. 3), thus suggesting a specific involvement of Shp-1 for Ret function. Therefore, as Shp-1 has no described intrinsic affinity to rafts compartments, the most likely explanation to reconcile these results is that binding to Shp-1 would happen outside rafts and then moves within rafts in complex with the active Ret molecules.

Therefore, given the inhibitory action of Shp-1 on Ret signaling [16], it seems likely that this phosphatase may contribute to generate a negative feedback mechanism to attenuate downstream signaling. If this is the case, the enrichment of Shp-1-bound Ret molecules (both Ret^{C634Y} and Ret^{M918T}) in rafts might be part of this negative mechanism. Even though, since Ret neither directly binds to Shp-1 nor it is a good substrate for this phosphatase (see Figs. 4 and 5), identifying the Shp-1 substrates in the Ret complex remains to be determined.

In conclusion, by using two constitutive active Ret mutants, here we report the implication of both Shp-2 and Shp-1 as components of signal transduction machinery that are associated to different membrane compartments thus mediating distinct functions of the receptor activity.

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Expression of GFR α 1 receptor splicing variants with different biochemical properties is modulated during kidney development

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Abstract

The glial cell line-derived neurotrophic factor (GDNF) family coreceptor α 1 (GFR α 1) is a critical component of the RET receptor kinase signal-transducing complex. The activity of this multicomponent receptor is stimulated by the glial cell line-derived neurotrophic factor (GDNF) and is involved in neuronal cells survival and kidney development. GFR α 1 pre-mRNA is alternatively spliced and produces two isoforms: GFR α 1a, which includes the exon 5; and GFR α 1b, which excludes it. Here we show that the GFR α 1a isoform is predominantly expressed in neuronal tissues and in PC12 cells differentiated toward a neuronal phenotype. GFR α 1 splicing is also regulated during kidney development, GFR α 1a is the minor isoform before birth and then rapidly becomes the major form after birth. We established cell lines expressing either GFR α 1 isoforms and demonstrated that the GFR α 1b isoform binds GDNF more efficiently than GFR α 1a. Consistently, GFR α 1b promotes a stronger RET phosphorylation than GFR α 1a. These results indicate that specific inclusion of the GFR α 1 exon 5 in neuronal tissues or during kidney development may alter the binding properties of GDNF to GFR α 1, and thus could constitute an additional regulatory mechanism of the RET signaling pathway.

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Keywords: GFR α 1; RET; Alternative splicing; Kidney development

1. Introduction

The glial cell line-derived neurotrophic factor (GDNF), Neurturin (NRTN), Persephin, and Artemin all belong to the GDNF family ligands [1]. These polypeptide growth factors support in vivo the survival of many types of neuronal cells both in the central and in the peripheral nervous system [2]. The biological actions of the GDNF family are mediated through a multicomponent receptor complex that contains a common signaling component, the RET receptor tyrosine kinase and one of the four members of the GFR α family (GFR α 1 to α 4). These glycosyl–phosphatidyl–inositol (GPI)-linked proteins are clustered in the lipid raft

structures of the extracellular cell membrane (reviewed in Ref. [3]). Converging evidence indicate that the GFR α s are necessary for at least three distinct functions: (i) the correct localization of the RET receptor on cell membrane rafts; (ii) the interaction between GDNF family ligands and RET; and (iii) the binding of GDNF to the NCAM receptor and the subsequent RET-independent signaling pathway activation [4].

GFR α 1, in conjunction with RET, binds to both GDNF and NRTN, with preferential pairing with GDNF [1,5]. GFR α 1-deficient mice have minor deficits in the central and peripheral nervous systems, but present profound deficits of the enteric nervous system formation. Moreover, they present an agenesis of the kidney due to the failure of the ureteric bud to emerge and to respond to growth signals from the metanephric mesenchyme [6,7], a phenotype similar to that observed in GDNF- and RET-deficient mice

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[2,8,9]. The human GFR α 1 protein (465 amino acids) is constituted of a signal peptide at the N-terminus, of a stretch of 20–30 hydrophobic amino acids at the C-terminus, and of three possible N-glycosylation sites [10,11]. The secondary structure predicts for the GFR α s an organization in three globular cystein-rich domains joined together by less-conserved hinge domains [3]. The human GFR α 1 gene has been mapped on chromosome 10, presents 11 exons spanned on 70 kbp, and is alternatively spliced [12].

Alternative splicing appears as a primary source of human proteomic diversity. Indeed, with $\sim 60\%$ of human genes expressing multiple mRNAs and $\sim 80\%$ of these alternative splicing events leading to variation in the encoded protein, alternative splicing provides an additional level of complexity to transducing pathway [13,14]. For instance, RET pre-mRNA alternative splicing leads to the production of two isoforms, RET9 and RET51, which possess different biochemical and biological properties [15–18]. The relative abundance of these isoforms varies during kidney development [16,19] and during tumorigenesis [20]. Several transcript variants have also been observed for the GDNF [8], GFR α 1 [21,22], GFR α 2 [9], and GFR α 4 [23–25] pre-mRNAs. GFR α 1 alternative splicing produces two isoforms, GFR α 1a and -b, which differ by the presence or the absence of the exon 5 [7,21,22]. The peptide stretch encoded by this exon is an inframe-conserved coding segment ($^{140}\text{DVFQQ}^{144}$) falling within the putative hinge region between GFR α 1 cystein-rich globular domains 1 and 2. Deletion studies have shown that the GFR α 1 central region (including the exon 5 segment) is essential for GDNF-binding efficiency suggesting that the presence or absence of the exon 5 might alter the binding properties of GDNF for GFR α 1 [6]. However, little is known about the regulation and the biological significance of GFR α 1 alternative splicing.

In this work, we examined the abundance of both GFR α 1 isoforms in several rat and human tissues. We observed that GFR α 1 alternative splicing is regulated in a tissue-specific way, as well as during kidney development. Examination of the biochemical properties of the two GFR α 1 isoforms revealed that they present different binding constants for the GDNF and NRTN ligands. Consistently, the GFR α 1b isoform, which binds GDNF and NRTN more efficiently (at low concentrations), is also found to be a better inducer of the RET autophosphorylation. These results suggest that the regulation of the GFR α 1 isoforms via alternative splicing may play a role in neuronal differentiation as well as during kidney development.

2. Materials and methods

2.1. RNA samples

Total RNA was isolated from embryonic and postnatal Sprague–Dawley rat tissues or Neuro-2a cells using RNA B

(Bioprobe systems). Various brain areas, including mesencephalon, cerebellum, bulb, were dissected from a pool of three to nine embryos and newborns under stereoscopic view. Polyadenylated RNA fractions extracted from kidney and substantia nigra were purified with Dynabeads mRNA DIRECT Kit (Dyna). Human RNA samples were purchased from Clontech. Three RNA samples of normal adrenal gland were used, (a.g.1) and (a.g.2) were two different pools of total RNA each extracted from six whole adrenal gland specimens; (a.g.3) was a pool of polyadenylated RNA extracted from 67 tissue specimens. The substantia nigra RNA sample (s.n.) was a pool of polyadenylated RNA extracted from 51 tissue specimens; the kidney RNA sample (kid.1) was a pool of total RNA extracted from two tissue specimens, (kid.2) was a sample of polyadenylated RNA purified from the kid.1 sample with Dynabeads mRNA DIRECT Kit (Dyna).

2.2. RNA probes and synthetic RNAs

Labeled antisense RNA probes and synthetic sense RNAs were transcribed from GFR α 1a and GFR α 1b DNA templates obtained by PCR (the downstream oligonucleotide includes the T7 promoter sequence) from human and rat GFR α 1a and GFR α 1b cDNAs, respectively. The human and the rat probes, Ph and Pr, contain each 76 nucleotides of exon 4, exon 5, and 119 nucleotides of exon 6 from human and rat GFR α 1a cDNAs, respectively. The rat-labeled antisense RNA probe P2 contains 38 nucleotides of exon 3, exon 4, exon 5 and 15 nucleotides of exon 6. Human and rat sense RNAs sR4/6 contain exon 4 and exon 6; sR4/5/6 contain exon 4, exon 5 and exon 6. Sense RNA probes were synthesized in 40 mM Tris–HCl pH 7.5, 10 mM NaCl, 6 mM MgCl₂, 2 mM spermidine, 6 mM DTT, 140 μ M ATP, 140 μ M GTP, 140 μ M CTP, 7 μ M UTP, 4 μ M [α -³²P]UTP (3000 Ci/mM, ICN), 2 pM of template DNA, 50 u of T7 RNA polymerase (BioLabs), in a total volume of 10 μ l incubated for 30 min at 37 °C, then incubated for 30 min with 10 u of DNase I (Boehringer Mannheim) and purified on 5% denaturing acrylamide gel. Synthetic sense RNA were synthesized and purified in the same conditions as RNA probes except for 0.6 mM ATP, 0.6 mM GTP, 0.6 mM CTP, 0.5 mM UTP, and 80 nM [α -³²P]UTP. RNA probes and sense RNAs were resuspended in H₂O containing 2 mg/ml of *Escherichia coli* tRNA.

2.3. RNases protection assays

Cellular RNAs or synthetic mRNAs and 0.2 fmole of labeling RNA probe were mixed, lyophilized, resuspended in 80% formamide, 0.4 M NaCl, 40 mM PIPES, 1 mM EDTA in a total volume of 7 μ l, heated for 5 min at 85 °C, incubated 12 h at 62 °C, digested by addition of 87 μ l of digestion buffer (300 mM NaCl, 10 mM Tris pH 7.5, 5 mM EDTA), 50 mg/ml RNase A (Sigma), 120 u/ml RNase T1 (Sigma), incubated 30 min at 30 °C, treated with 0.1 mg/ml

proteinase K (Boehringer Mannheim) and 0.7% SDS for 30 min at 37 °C, phenol extracted, precipitated with ethanol, and analyzed on 7% acrylamide denaturing gel. The measurements were performed with a PhosphorImager (Molecular Dynamics). RNA-protected bands obtained with synthetic RNAs were used as standards to calculate the absolute amounts of the corresponding mRNA species contained in the cellular RNA samples. In all experiments, the intensities of the bands of interest increased linearly with the amount of cellular RNA.

2.4. RT-PCR assays

Reverse transcription was performed with 2 µg of total RNA heated at 94 °C for 2 min and incubated 1 h at 42 °C in 50 mM NaCl, 50 mM KCl, 10 mM Tris–HCl pH 8, 6 mM MgCl₂, 1 mM EDTA, 10 mM DTT, 0.5 mM of each dNTP, 2 mM random hexanucleotides (Boehringer Mannheim), 10 u of AMV reverse transcriptase (Boehringer Mannheim). Amplification was carried out with 1:5 of the RT reaction in 20 mM Tris–HCl pH 8.4, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1 mg/ml BSA, 0.1% triton X100, 0.2 mM dNTPs, 10 nM [α -³²P]CTP (800 Ci/mM, ICN), 1.2 u of Pfu DNA polymerase (Stratagene), and 0.5 µM of the following primers, GATATATTCCGGG-CAGTCCCGTTC and CCTGTGCGAAGAACTGCCT-GAGG. The resulting 195 and 210 bp DNA fragments were phenol extracted, precipitated with ethanol, and analyzed on 7% acrylamide denaturing gel. Measurements were performed with a PhosphorImager.

2.5. Cell line and transfection assays

The NGR-38 clone is a rat GFR α 1a-expressing Neuro-2a cell line already described [10,26]. The GFR α 1b expression vector was ingenerated by inserting into the GFR α 1a expression vector PSJA45 [10] a DNA fragment lacking the exon 5 obtained by PCR from a rat GFR α 1b plasmid and kindly provided by M. Billaud (Lyon, France). The resulting vector was stably transfected using Eugene 6 (Boehringer Mannheim) into Neuro-2a cells leading to GFR α 1b-expressing clones. All GFR α 1a or GFR α 1b Neuro-2a cell lines were grown in RPMI 1640 (Gibco BRL), 10% fetal calf serum, 200 µg/ml G418 (Sigma), and assayed for GFR α 1 expression and GDNF binding (data not shown). As NGR-38 (N2 α 1a) and N2 α 1Short1 (N2 α 1b) clones express similar quantities of GFR α 1 and respond physiologically to GDNF treatment, they were elected for further experiments.

2.6. Ligand binding

Recombinant GDNF and NRTN and radio-labeled GDNF and NRTN were prepared as described previously [10,26]. Binding of [¹²⁵I]GDNF to cells expressing GFR α 1a or GFR α 1b was carried out as described previously [26].

Briefly, cells expressing GFR α 1a or GFR α 1b were seeded at a density of 0.4×10^6 cells per well in 24-well tissue culture plates (Becton-Dickinson) precoated with polyornithine 24 h prior to use. Cells were placed on ice for 510 min, washed once with ice-cold washing buffer (DMEM containing 25 mM HEPES, pH 7.5), and then incubated with 50 pM [¹²⁵I]GDNF in binding buffer (washing buffer plus 0.2% bovine serum albumin) at 4 °C for 4 hr, either in the presence or absence of indicated concentration of unlabeled GDNF or NRTN. Cells were then washed four times with ice-cold washing buffer and lysed in 1 M NaOH. The radioactivity associated with the cells was determined in a 1470 Wizard Automatic Gamma Counter (Wallac).

2.7. Immunoblotting analysis

The autophosphorylation of RET was examined by immunoblot analysis as described previously [10,26]. Briefly, cells were seeded in 6-well tissue culture plates at a density of 1.5×10^6 cells/well 24 h prior to use. Cells were washed once with binding buffer and treated with various concentration of GDNF or NRTN (0.01 to 250 ng/ml) in binding buffer at 37 °C, for 10 min. Treated cells were lysed in Triton X100 lysis buffer (50 mM HEPES, pH 7.5, 1% Triton X100), 50 mM NaCl, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% aprotinin (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma), 0.5 mM Na₃VO₄ (Fisher), and immunoprecipitated with anti-RET antibodies (Santa Cruz Biotechnology) and protein A-Sepharose [10]. Immunoprecipitates were fractionated by 7.5% SDS-PAGE and transferred to nitrocellulose membranes. The membrane were blocked with 5% bovine serum albumin (Sigma), and the level of tyrosine phosphorylation of RET was determined by probing the membrane with an antiphosphotyrosine monoclonal antibody, 4G10 (UBI), at room temperature for 2 h. The amount of RET protein in each lane was determined by stripping and reprobing the same membrane with the anti-RET antibody. Detection was accomplished using a sheep antimouse secondary antibody directly conjugated to horseradish peroxidase (Amersham) in conjunction with chemiluminescence reagents (ECL, Amersham). The ECL data were analyzed by using Eagle Eye (Stratagen) following the manufacturer's instruction.

2.8. Erk-1 immunokinase assay

Proteins lysates (400 µg) from GFR α 1a- or GFR α 1b-expressing Neuro-2a cell lines were incubated 2 h at 4 °C in 200 µl of lysis buffer [10 mM Tris–HCl pH 8, 150 mM NaCl, 0.4 mM EDTA, 1% NP40, 10 mM NaF, 10 mM Na₂H₂P₂O₇, 2 mg/ml aprotinin (Roche), 2 mg/ml leupeptin (Roche), 100 mg/ml AEBSF, 2mM Na₃VaO₄] and 0.4 µg of anti-Erk-1 antibody C16 (Santa Cruz Biotechnology) previously bound to protein A-Sepharose (Amersham), then washed 5 min in 500 µl lysis buffer and twice in assay buffer (20 mM HEPES pH 7.5, 10 mM MgAc, 2 mg/ml

Fig. 1. GFR α 1 exon 5 alternative splicing pattern. (A) Sequence of the human GFR α 1 gene including exon 4,5, and 6 (bold) and neighbour intronic regions. (B) Splicing pattern of the GFR α 1 pre-messenger RNA. The boxes and lines outline the exons and introns, respectively (not drawn to scale).

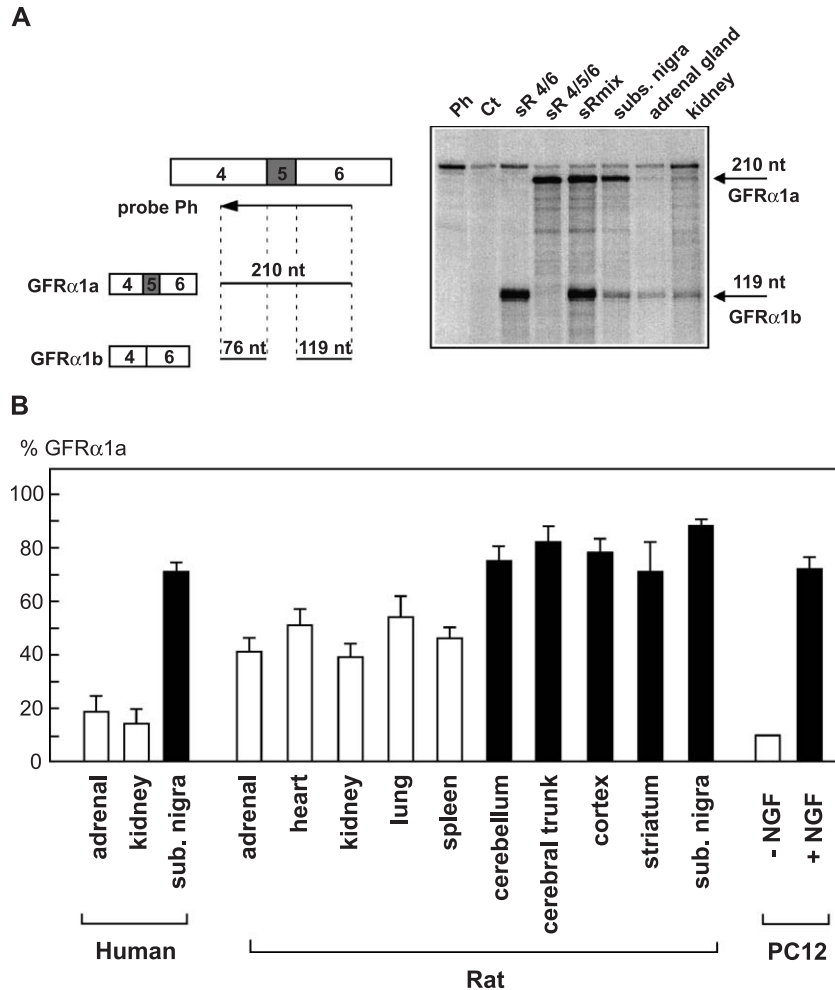


Fig. 2. Tissue-specific alternative splicing of GFR α 1 exon 5. Total RNA samples or poly A fractions of adult human and rat tissues were analyzed by RPA. (A) Analysis of human tissues. Ph: nondigested probe; Ct: digested probe; sR4/6 and sR4/5/6: 3 amoles of synthetic transcript containing either exon 4, 5, and 6 or exon 4 and 6, respectively; sRmix: 3 amoles of an equimolar mixture of sR4/6 and sR4/5/6; subs.nigra: 0.4 μ g of substantia nigra poly A RNA; adrenal gland: 0.4 μ g of adrenal gland poly A RNA; kidney: 0.4 μ g of kidney poly A RNA. (B) Percentage of the GFR α 1a transcript species in human and rat tissues and in PC12 cells before and after treatment with NGF. Black box: neuronal tissues and NGF-treated cells; open box: others. The proportion of GFR α 1a referred to the sum of both GFR α 1 transcripts and is given as mean \pm S.E.M. corresponding to three or more experiments.

RNA was extracted from rat tissues and was analysed by quantitative RT-PCR assays (Fig. 3A). GFR α 1a was the major isoform present in several neuronal structures (cortex, mesencephalum, cerebral trunk, cerebellum). In these tissues, the proportion of GFR α 1a presented little variation during development (ranging between 70% and 85%; Fig. 3A). On the contrary, in kidney, GFR α 1a represented 43% to 46% of all GFR α 1 transcripts at E18 and P0. This amount raised to $67 \pm 2\%$ at P3, P6, P10, thus showing a significant induction immediately after birth. This value then declined to $40 \pm 4.2\%$ in adult. This observation was verified by RNases protection assays performed with a probe spanning rat GFR α 1 exon 3 to exon 6. As shown in Fig. 3B, the proportion of GFR α 1a raised from 38% at P0 to 57% after birth (P3), thus confirming the RT-PCR data. In conclusion, both RPA and RT-PCR experiments show a rapid increase of GFR α 1 exon 5 inclusion (GFR α 1a) after birth in rat kidney.

3.2. Functional studies of the GFR α 1 isoforms

Taken together, the results presented above suggest that the exon 5 inclusion in the GFR α 1 transcript may modulate the cell-specific response to GDNF or NRTN stimulation. To compare the ability of each GFR α 1 isoforms to mediate RET stimulation, we generated cell lines that stably expressed either the GFR α 1a or the GFR α 1b isoform. Neuro2-a cells, a mouse neuroblastoma cell line that expresses high levels of RET but no detectable levels of GFR α 1 mRNA [10], were transfected with either GFR α 1a or GFR α 1b cDNA expression vector. We selected clones (N2 α 1a and N2 α 1b cells) that express similar quantities of GFR α 1 and respond physiologically to GDNF treatment (data not shown). We determined the induction of neurite outgrowth in N2 α 1a and in N2 α 1b cells. Although the GDNF treatment caused a change in the cell morphology characterized by an extended neurite outgrowth, the extent

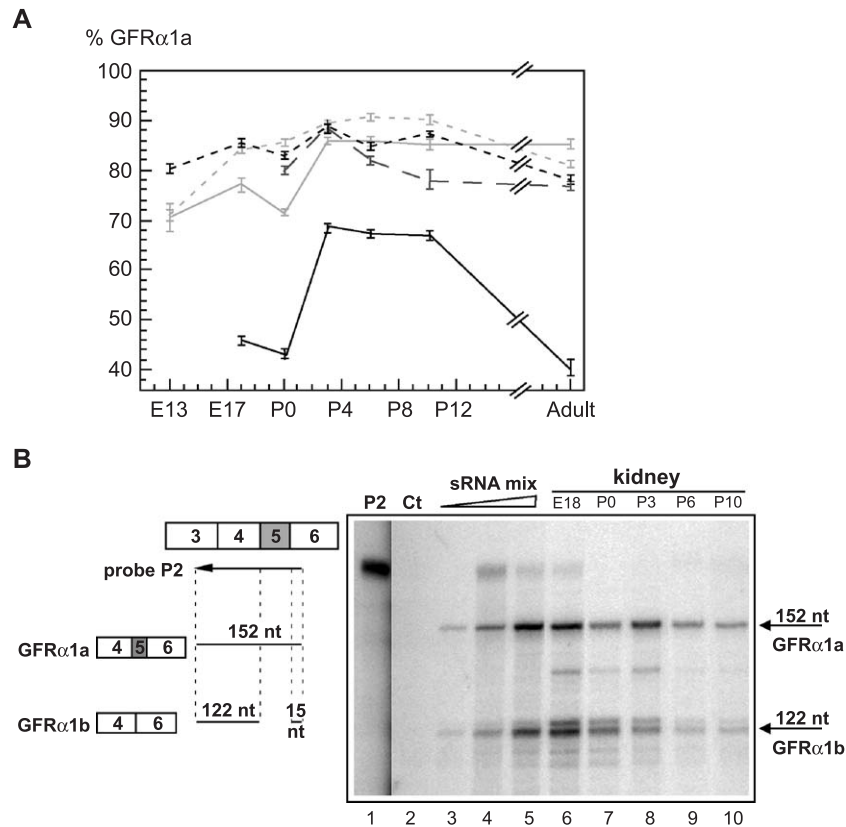


Fig. 3. GFRα1 exon 5 alternative splicing during development. Total RNA was extracted from rat tissues and was analyzed by RT-PCR and RPA. (A) Proportion of GFRα1a transcript analyzed by RT-PCR. Black line: kidney; dotted black line: cortex; light gray line: mesencephalum; dotted light gray line: cerebral trunk; dotted dark gray line: cerebellum. The proportion of GFRα1a referred to the sum of both GFRα1 transcripts (mean \pm S.E.M.) corresponding to three experiments. (B) RPA analysis performed on the same RNA samples than in (A). P2: nondigested probe; Ct: digested probe; lanes 3–5: 1, 3, and 9 amoles of an equimolar mixture of sR4/6 and sR4/5/6; lanes 6–10: 3 μ g of kidney RNA corresponding to the indicated days before and after birth (E18, P0, P3, P6, P10).

of these networks was comparable between GFRα1a- or GFRα1b-expressing cells (data not shown). Similar results were obtained upon stimulation with NRTN.

3.2.1. GFRα1 isoforms differ in GDNF-binding affinities

We examined if the two GFRα1 isoforms present different ligand-binding affinities to either GDNF or NRTN. GFRα1a- or GFRα1b-expressing cells were incubated with [125 I]GDNF in the absence or presence of excess unlabeled GDNF or NRTN. As shown in Fig. 4, a high-affinity and a low-affinity GDNF/ NRTN binding sites could be distinguished in GFRα1-expressing cells. Consistent with previous observations [10], the high-affinity site binds GDNF with a K_d constant in the sub-pM range, while the low-affinity site presents a K_d constant around several hundred pM. In agreement with the preferential binding of GFRα1 to GDNF, the affinities of both the high- and the low-affinity binding sites for GDNF were several thousand-fold larger than for NRTN.

Importantly, our results show that, between 10^{-5} and 10^{-1} pmol of cold ligand, [125 I]GDNF binding competition by nonlabeled GDNF (Fig. 4A) or NRTN (Fig. 4B) is higher when cells express the GFRα1b isoform than when they

express the GFRα1a form. This indicates that at low concentrations of the cold ligand, both GDNF and NRTN bind to the GFRα1b isoform more efficiently than to the GFRα1a form, while at larger concentrations, less significant differences could be observed. Thus, the high-affinity site of the GFRα1b isoform (excluding the exon 5) presents an affinity for GDNF and NRTN larger than that of the GFRα1a isoform. On the opposite, the low-affinity monomeric receptors of both isoforms behaved similarly.

3.2.2. GFRα1 isoforms mediate RET stimulation with different efficiency

We next investigated the capacity of each GFRα1 isoform to activate the RET receptor. GFRα1a- or GFRα1b-expressing cells were treated with various concentrations of GDNF or NRTN, followed by cells lysis, immunoprecipitation with an anti-RET antibody, and immunoblotting with an antiphosphotyrosine antibody. A 170-kDa band corresponding to phosphorylated RET was observed, with clear stimulation after GDNF or NRTN treatment. In agreement with the preferential binding of GFRα1 to GDNF, RET phosphorylation was three times larger with GDNF than with NRTN. The filters were next stripped and reprobated using the anti-RET

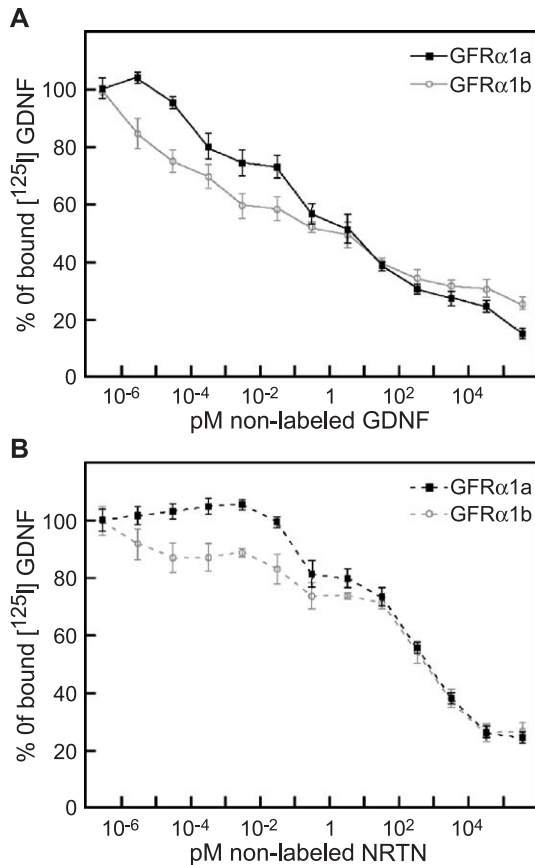


Fig. 4. GDNF and NRTN affinities for the GFR α 1 isoforms. [125 I]-labeled GDNF was added to GFR α 1a- or GFR α 1b-expressing cells and was then competed by the indicated concentrations of cold ligand. (A) Competition by nonlabeled GDNF. (B) Competition by nonlabeled NRTN. Black squares: GFR α 1a-expressing cells; grey circles: GFR α 1b-expressing cells; line: nonlabeled GDNF competition; dotted line: nonlabeled NRTN competition. The proportion of labeled GDNF bound to GFR α 1a- or GFR α 1b-expressing cells refers to the amount in the absence of unlabeled ligand and is given as mean \pm S.E.M.

antibody. Quantifications of the ratio phosphotyrosine/RET signals are presented in Fig. 5. At ligand saturation, RET activation was maximal and no difference between the GFR α 1 isoforms was observed. But, importantly, at low ligand concentrations GFR α 1b mediated RET phosphorylation at larger extent than GFR α 1a. For instance, at concentrations of 0.01, 0.1, and 1 ng/ml of GDNF, the RET activation was 5.8, 1.7, and 1.6 larger with GFR α 1b than with GFR α 1a, respectively (Fig. 5). Comparable ratios were observed with NRTN (4.3-, 3.3-, and 1.6-fold difference upon 0.01, 0.1, and 1 ng/ml NRTN treatment). Thus, consistent with its greater affinity for GDNF and NRTN, GFR α 1 isoform excluding the exon 5 (GFR α 1b) promotes a larger activation of the RET-dependent signaling pathway in tissues where GFR α 1b is the major variant.

On the other hand, we cannot exclude that RET-independent binding sites of GDNF to GFR α 1 may also contribute to the higher affinity of GDNF to the GFR α 1b

isoform (see Fig. 4). Indeed, recently, GFR α 1 has been shown to promote, in a RET-independent manner, GDNF binding to the NCAM receptor followed by activation of the Fyn/Fak signaling pathway [4]. Therefore we determined the levels of Fak Tyr-397 phosphorylation and the Fyn activity in both the N2 α 1a and N2 α 1b cells as indication of RET-independent intracellular signalling [4]. In both cell lines, GDNF stimulation (up to 150 nM for 10 min) was unable both to activate Fyn and to induce Fak Tyr-397 phosphorylation (data not shown), thus indicating that the RET-independent binding of GDNF to GFR α 1 unlikely contributes to the GDNF-binding affinities differences between GFR α 1a- and GFR α 1b-expressing cell lines.

3.2.3. GFR α 1 isoforms mediate similar Erk activation

Upon GDNF stimulation, RET autophosphorylation leads to the activation of downstream signaling, such as the Ras/Erk pathway essential for PC12 cells neuronal differentiation [27]. We measured the GFR α 1-mediated effects of GDNF and NRTN on the activation of the downstream effectors Erk kinases. GFR α 1a- or GFR α 1b-expressing cells were treated with various concentrations of GDNF or NRTN, followed by cells lysis, immunoprecipitation with an anti-Erk1 antibody, and kinase activity assay using the Myelin Basic Protein (MBP) as a substrate (Fig. 6A). MBP phosphorylation quantification in response to GDNF or NRTN treatment kinetics and dose-responses are presented in Fig. 6B and C, respectively. No significant differences were observed between N2 α 1a- and N2 α 1b-treated cells. Note that consistent with the preferential binding of Gfr α 1 to GDNF than to NRTN, the MBP phosphorylation kinetics was delayed of about 4 min upon NRTN treatment as compared to GDNF treatment. Overall,

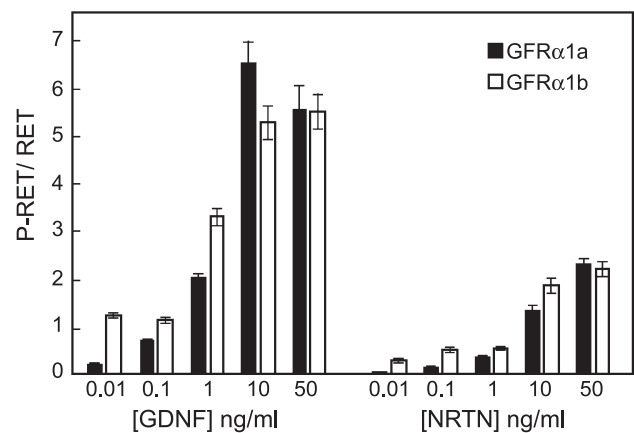


Fig. 5. GFR α 1b promotes RET phosphorylation more efficiently than GFR α 1a. GFR α 1a- or GFR α 1b-expressing cells were treated with the indicated concentrations of GDNF or NRTN. Cells were lysed, and RET phosphorylation was analysed by RET immunoprecipitation followed by antiphosphotyrosine immunoblotting, quantification, stripping, anti-RET immunoblotting, and quantification. The ratio of phosphotyrosine signal/RET signal was calculated after subtraction of the values in absence of ligand and is given as mean \pm S.E.M. Black box: GFR α 1a-expressing cells; open box: GFR α 1b-expressing cells.

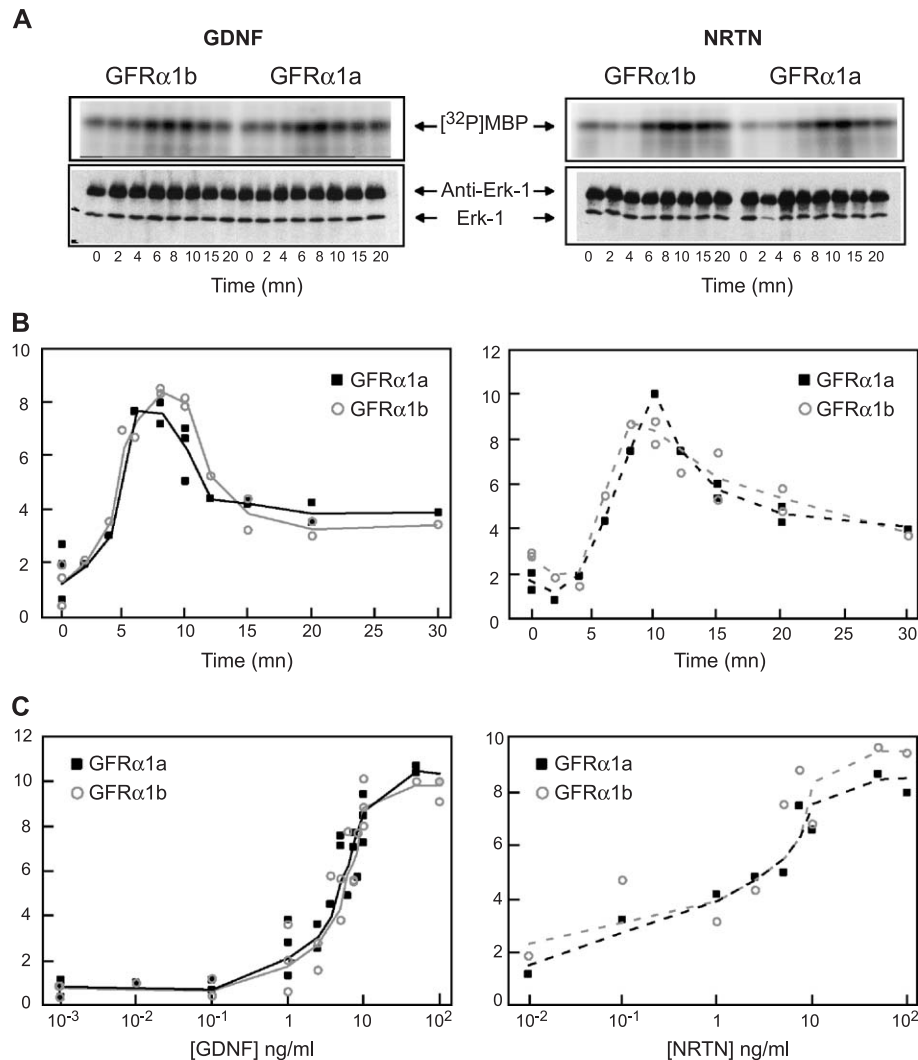


Fig. 6. Erk activation promoted by GFRα1a and GFRα1b isoforms. Serum-starved GFRα1a- or GFRα1b-expressing cells were treated for the indicated times and with the indicated concentrations of either GDNF (left panel) or NRTN (right panel). (A) Cell lysates were immunoprecipitated with anti-Erk-1 antibody and subjected to immunokinase assay using the exogenous substrate Myelin Basic Protein (upper panel). The amounts of immunoprecipitated Erk-1 was checked by immunoblot with anti-Erk-1 antibody (lower panel). [³²P]MBP: phosphorylated Myelin Basic Protein; Anti-Erk-1: heavy molecular chain of the anti-Erk-1 immunoprecipitating antibody. (B) Kinetics of MBP phosphorylation in response to 100 ng/ml GDNF (left) or NRTN (right) treatment. (C) Dose responses of MBP phosphorylation in response to 6 mn GDNF (left) or 10 mn NRTN (right) treatment. Numbers in ordinate refers to the fold activation of MBP phosphorylation in the absence of treatment. Black squares: GFRα1a-expressing cells; grey circles: GFRα1b-expressing cells; line: GDNF treatment; dotted line: NRTN treatment.

these results indicate that despite a stronger phosphorylation of RET, the GFRα1b isoform did not mediate a consistent higher Erk-1 activity as compared to GFRα1a. A possible interpretation for this apparent discrepancy may rely on the balance acting on Erks between the positive stimulation initiated by the extracellular signal and the reverse dephosphorylation reactions that would compensate the differences observed for RET activation [28,29].

4. Discussion

Genome-wide analyses of alternative splicing estimate that 40% to 60% of human genes are subject to alternative

splicing, and that over three-quarters of these splicing isoforms may be involved in cell signaling and cell regulation, suggesting that alternative splicing is a significant component of the human genome functional complexity [13,14]. For instance, alternative splicing can alter the ligand-binding affinity of the fibroblast growth factor receptor-2 [30] or the substrate specificity of the protein tyrosine kinases src [31], Fak [32], and RET [33]. Here, we studied the tissue-specific expression and the biochemical properties of two isoforms produced by alternative splicing of the GFRα1 coreceptor pre-mRNA.

We first showed that the regulation of the GFRα1 pre-mRNA alternative splicing leads to the overproduction of the GFRα1a isoform (including exon 5) in many neuronal

compared to nonneuronal tissues. Similar expression patterns were observed in human as well as in rat tissues. In agreement with these observations, we showed that PC12 cells, if differentiated toward a neuronal phenotype by NGF treatment, preferentially expressed the GFR α 1a variant. Whether the predominance of this isoform in neuronal structures may mediate cell-specific transducing effects initiated by RET remains to be determined.

During early development, we observed that the relative expression of the GFR α 1a variant increased rapidly after birth in the rat kidney but not in brain structures, where it remained expressed at high levels. A similar developmental pattern has been previously observed for the RET-splicing regulation. Alternative splicing of the RET pre-mRNA 3' end results in two variants, the RET9 and the RET51 isoforms that present different biochemical and biological properties [15,17,34–36]. Study of developing human kidney showed that the relative expression of the RET51 isoform increased during early development [19]. Such similarities between RET and GFR α 1 splicing regulation support the possibility that alternative splicing alteration of the GDNF-induced RET transducing pathway may play an important role in kidney development. Whether this tissue-specific regulation of GFR α 1 and RET splicing pattern is coordinated by common effectors or results from independent activators is currently unknown. Furthermore, distinct splice variants are also observed for GDNF, GFR α 2, and GFR α 4 pre-mRNA. Some of the GFR α 4-splicing isoforms are functional and are produced in tissue-specific and developmentally regulated processes, suggesting possible roles in endocrine cell development and in MEN2 tumorigenesis [24,25]. Thus, coordination of the alternative splicing regulation of the different components of the RET-transducing pathway may provide an additional level for cell-signaling complexity and specificity.

The existence of the two GFR α 1-splicing isoforms, as well as the GFR α 1 exon 5 peptide sequence (¹⁴⁰DVFQQ¹⁴⁴), is conserved between mouse, rat, and human but not among the other members of the GFR α family, suggesting a specific alternative splicing regulation of the GFR α 1 cell-signaling pathway. Moreover, GFR α 1 exon 5 is localized in the hinge region between two putative cystein-rich domains, immediately upstream of the boundary of domain 2. Deletion and modeling studies indicate that this domain determines the ligand-binding specificity of the GFR α receptors and is required for the RET/GFR α 1 association [6,37,38]. Thus, the length and the sequence of the hinge region may be important for the flexibility of the protein and for its interactions with the ligand and/or with RET. Our studies of GFR α 1a- or GFR α 1b-expressing cells support this hypothesis, as the high-affinity site of the GFR α 1b isoform (excluding the exon 5) presents a larger affinity for GDNF and NRTN than that of the GFR α 1a isoform. Consistently, the higher RET phosphorylation mediated by the GFR α 1b isoform compared to the GFR α 1a form well reflects the ligand- (either GDNF or NRTN) binding properties of the

two isoforms. Furthermore, absence of Fyn/Fak activation in N2 α 1a or N2 α 1b cells after GDNF treatment indicates that RET-independent binding of GDNF to GFR α 1 unlikely takes place in our conditions. The observation that a higher RET activity did not induce higher levels nor different kinetics of Erk stimulation likely reflects the complexity of the downstream RET-induced signaling in different cell systems [28].

Taken together, these observations allow us to propose that the predominant inclusion of exon 5 in the GFR α 1 pre-mRNA, in neuronal tissues or during kidney development, constitutes an additional regulation mechanism of the RET-signaling pathway.

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Direct interactions among Ret, GDNF and GFR α 1 molecules reveal new insights into the assembly of a functional three-protein complex

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Abstract

The glial-cell-line-derived neurotrophic factor (GDNF) ligand activates the Ret receptor through the assembly of a multiprotein complex, including the GDNF family receptor α 1 (GFR α 1) molecule. Given the neuroprotective role of GDNF, there is an obvious need to precisely identify the structural regions engaged in direct interactions between the three molecules. Here, we combined a functional approach for Ret activity (in PC12 cells) to cross-linking experiments followed by MS-MALDI to study the interactions among the purified extracellular region of the human Ret, GDNF and GFR α 1 molecules. This procedure allowed us to identify distinct regions of Ret that are physically engaged in the interaction with GDNF and GFR α 1. The lack of these regions in a recombinant Ret form results in the failure of both structural and functional binding of Ret to GFR α 1/GDNF complex. Furthermore, a model for the assembly of a transducing-competent Ret complex is suggested.

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Keywords: Tyrosine kinase receptor; MALDI mass spectrometry; GDNF; Ret

1. Introduction

The *ret* protooncogene encodes a tyrosine kinase receptor that plays a crucial role in kidney morphogenesis and in the survival and differentiation of several subpopulations of neurons in the peripheral and central nervous systems [1]. The Ret protein consists of three functional

regions: the extracellular region, including four N-terminal cadherin-like domains (named CLD1 to CLD4) followed by a single cysteine-rich domain (CRD), the transmembrane region and the intracellular region formed by a bipartite tyrosine kinase domain [2].

Four members of the glial cell line-derived neurotrophic factor (GDNF) family, including GDNF, neurturin, artemin and persephin represent the Ret ligands. Ret activation by these neurotrophic factors is mediated by their binding to one of the four different glycosyl phosphatidylinositol-anchored receptors, termed GDNF family receptor α (GFR α) 1 to 4 [3]. GDNF has potent trophic effects on dopaminergic nigral neurons, indicating this factor as a promising protective agent in neurodegenerative diseases. In various animal models of Parkinson's disease, GDNF has been shown to prevent the neurotoxin-induced death

Abbreviations: CLD, cadherin-like domain; CRD, cysteine-rich domain; GDNF, glial cell line-derived neurotrophic factor; GFR α 1, GDNF family receptor α 1; EC-Ret^{wt}, Ret extracellular portion; EC-Ret^{1–387}, Ret extracellular portion deleted of CLD4 and CRD.

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of dopamine neurons and can promote functional recovery (for a review, see Ref. [4]). Despite a cross-talk between the different ligands–GFR α s pairs, a preferred coreceptor molecule exists for each ligand, GDNF being the preferred high-affinity ligand for GFR α 1. On the other hand, in the absence of GFR α 1, Ret is not able to bind GDNF, whereas it can interact weakly with GFR α 1 even in the absence of GDNF [5–7]. GDNF-dependent activation of Ret implicates its recruitment to the GFR α 1/GDNF complex, leading to Ret dimerisation and autophosphorylation at specific cytoplasmic tyrosine residues [1]. Several structural elements in both GDNF and GFR α 1 that are required for the formation of the complex have been tentatively defined [8–10]. By constructing a set of chimeric and truncated coreceptors and analysing their ligand binding and signaling capabilities, a central domain in the GFR α 1 molecule was identified as necessary for physical and biochemical interaction with both GDNF and Ret molecules [11]. The crystal structure of the second half of this central domain has been recently solved, revealing it as an independent folding unit [12]. In addition, homologue-scanning mutagenesis of GDNF allowed identifying residues located along the second finger of the ligand according to the crystal structure as critical for the interaction with GFR α s molecules [8,9]. On the other hand, by constructing chimeric molecules constituted by the human and the *Xenopus* regions of the extracellular domain of Ret fused together, the binding determinants for GFR α 1/GDNF complex have been found to be mainly concentrated in the first CLD1 of Ret [13]. However, the identification of regions and specific residues directly engaged in the intermolecular interactions among the Ret, GDNF and GFR α 1 molecules and needed to form a functionally active transducing complex is still questioned.

This study is intended to identify key amino acids engaged in direct interactions between the three molecules. Nowadays, cross-linking reagents are widely used in the assessment of contact regions in protein–protein interaction mainly in combination with mass spectrometry as the analytical methodology able to precisely identify covalently linked residues [14–17].

Therefore, we used a proteomic-based approach to study the interactions among the pure extracellular region of human Ret, the GDNF and the GFR α 1 molecules. The three proteins were incubated *in vitro* and treated with a limited excess of cross-linking reagents. The reaction products were fractionated on SDS polyacrylamide gel (SDS-PAGE), and the individual cross-linked species were characterised by mass spectrometry fingerprinting analysis. The obtained results led to the identification of distinct regions in the extracellular region of Ret that are required for the assembly of a transducing-competent three-component complex. Signal transduction experiments in PC12 cells fully confirmed the structural analyses findings of the used approach.

2. Materials and methods

2.1. Cloning, expression and purification of EC-Ret proteins

The entire Ret receptor extracellular portion (EC-Ret^{wt}) was produced as previously reported [18]. The protein containing the first three N-terminal CLDs of EC-Ret^{wt} but lacking CLD4 and CRD (named EC-Ret^{1–387}) was produced as follows.

A fragment encoding residues 1–387 of EC-Ret^{wt} was amplified using Taq polymerase in standard PCR conditions (F: 5'AGTGTTTTAAATTTAAGCTTGCGGCCG3' and R: 5'CACATTCAAATGTAGTAAGGATCCGC3'). The C-terminus of the resulting PCR fragment was ligated to a fragment containing a tobacco etch virus (TEV) protease cleavage site followed by the two IgG binding domains of *S. aureus* protein A into a pcDNA 3.1 (+) expression vector (Invitrogen). The resulting plasmid was transiently transfected in HEK 293T cells at 90% confluence plated on to six 15-cm plates using the Lipofectamine 2000 reagent (Invitrogen). The ultrafiltrated culture medium (15 ml final volume) was loaded onto an IgG Sepharose 6 Fast Flow column (Amersham-Pharmacia, 0.5×3 cm), and the sample was eluted in the presence of TEV protease. The recovered sample was fractionated onto a Superdex 200 High Load column (Amersham-Pharmacia, 1×30 cm) connected to a Fast Protein Liquid Chromatography (FPLC) system. The total protein yield was approximately 500 ng/ml of culture medium.

2.2. Cell culture, preparation of cell extracts and immunoblotting analysis

PC12/wt (PC12 cells stably transfected with human Ret^{wt} receptor) and PC12- α 1/wt (PC12 cells stably transfected with both human Ret^{wt} receptor and GFR α 1 coreceptor) cells were grown as previously reported [19]. When indicated, GDNF (Promega) or recombinant rat GFR α 1-Fc chimera (R&D System) were added to the culture medium.

Cells were washed twice in ice-cold PBS then lysed in 50 mM Tris–HCl, pH 8.0 buffer containing 150 mM NaCl, 1% Nonidet P-40, 2 μ g/ml aprotinin, 1 μ g/ml pepstatin, 2 μ g/ml leupeptin, 1 mM Na₃VO₄. The solution was centrifuged at 16,000 g for 30 min at 4 °C, and the residue was discarded. Protein concentration was determined by the Bradford assay using bovine serum albumin as standard. After SDS-PAGE, proteins were electroblotted to polyvinylidene difluoride membranes (Millipore) and detected with the indicated primary antibodies and peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system (Amersham-Pharmacia). The primary antibodies used were the following: anti-Ret (H-300) and anti-ERK1 (C-16; Santa Cruz Biotechnology), anti-phosphoRet (Cell Signaling), anti-phospho44/42 MAP Kinase monoclonal antibodies (E10; Cell Signaling). When indicated, membranes were

stripped in 62.5 mM Tris–HCl pH 6.7, 0.1 M 2-mercaptoethanol, 2% SDS, for 30 min at 55 °C. The immunoblots shown are examples of at least three independent experiments.

2.3. Neurite outgrowth bioassay

PC12- α 1/wt cells were plated at equal density on 12-well culture plate. To evaluate the effects of EC-Ret proteins on cell differentiation, cells were incubated with 50 ng/ml GDNF together with the appropriate protein at a final concentration of 10 μ g/ml. At least 15 random fields were photographed 24 h after GDNF stimulation by using a phase-contrast light microscope, and 30 cells per frame were counted and scored as having neurites or not. A neurite was operationally defined as a process outgrowth that was long more than twice the diameter of cell body.

2.4. Deglycosylation procedure

Treatment with peptide N-glycosidase F (PNGase F) or endoglycosidase H (Endo H) was performed according to the protocol supplied by the manufacturer (New England Biolabs).

2.5. Chemical modifications

Cross-linking reactions were carried out in a total volume of 80 μ l of 10 mM sodium phosphate buffer, pH 7.5. Recombinant GDNF, GFR α 1 and EC-Ret^{wt} or EC-Ret^{1–387} were mixed in equimolar amount (2×10^{-6} mol/l) and incubated at 25 °C for 30 min. Following complex formation, either suberic acid bis (3-sulpho-*N*-idroxysuccinimide ester) sodium salt (BS3) or 1-ethyl-3-(3-dimethylamino propyl)-carbodiimide (EDAC) cross-linking reagents (Sigma) were added to the mixture. Several preliminary experiments were carried out to precisely determine the optimal excess of chemical reagents. A molar excess of 1:2500 (mol/mol) for both reagents was eventually defined, and the modification reactions were allowed to proceed at 25 °C for 3 h.

2.6. SDS-PAGE analysis and “in situ” digestion

Cross-linking reactions were terminated by quenching the excess of reagents with 20 μ l of gel loading buffer (Tris–HCl 180 mM, 6% SDS, 30% Glycerol, Blu Bromophenol 0.03%, pH 6.8). Samples were heated at 100 °C for 5 min and then separated by gel electrophoresis through a 7% tricine SDS polyacrylamide gel. Proteins were detected by silver staining, and positively stained protein bands were excised from the gel and washed in deionised MilliQ grade water (2 times, 10 min). The excised spots were destained with 100 μ l of destaining solution (30 mM K₃Fe(CN)₆, 100 mM Na₂S₂O₃) and washed with 100 μ l of 100 mM ammonium bicarbonate buffer. After 5 min, an equal

amount of acetonitrile was added. Protein samples were reduced by incubation in 10 mM dithiothreitol (DTT) for 45 min at 56 °C. Reducing solution was removed by acetonitrile wash, and free cysteines were alkylated by treatment with 55 mM iodoacetamide for 30 min at room temperature in the dark. The supernatant of the alkylating solution was discarded, and the reaction was stopped by washing gel pieces with ammonium bicarbonate buffer and acetonitrile. Enzymatic digestion was carried out by incubating gel pieces with 30 μ l of a 1 ng/ μ l trypsin solution in 10 mM ammonium bicarbonate at 4 °C for 2 h. The excess of trypsin solution was then removed, and a new aliquot of 10 mM ammonium bicarbonate was added; samples were incubated for 18 h at 37 °C. A minimum reaction volume was used to obtain the complete hydration of the gel. Peptides were then extracted by washing the gel particles with 0.1% TFA in 50% acetonitrile at room temperature. Peptides were purified by using ZipTip Pipette Tips following manufacturer protocol (Millipore Billerica, USA).

2.7. MALDI-MS analyses

MALDI-TOF mass spectra were recorded using an Applied Biosystem Voyager DE-PRO instrument. A 1- μ l aliquot of a mixture of analyte and matrix solution (alpha-cyano-hydroxycinnamic acid 10 mg/ml in 66% ACN, 0.1% TFA, in MilliQ water) was applied to the metallic sample plate and dried down at room temperature. Mass calibration was performed using external peptide standards. Raw data were analysed using the computer software provided by the manufacturer and reported as monoisotopic masses. The accuracy of the mass determinations was always within 30 ppm. The percentage of noninterpreted signals was less than 10%.

3. Results

3.1. Production of EC-Ret^{wt} and EC-Ret^{1–387}

To perform cross-linking experiments, we took advantage of pure protein preparations corresponding to the entire Ret extracellular region, protein ending at amino acid 636 (EC-Ret^{wt}) [18], and of a deletion mutant encompassing CLD1, two and three of the native protein but lacking CLD4 and CRD (EC-Ret^{1–387}). The recombinant Ret truncated form was produced as described in Materials and methods section and characterised for its homogeneity. An aliquot of 30 μ g of purified EC-Ret^{1–387} loaded onto a Superdex 200 (FPLC System) gave rise to a symmetric chromatographic peak with an apparent molecular mass of approximately 80 kDa, which was compatible with a homogeneous preparation of the protein in a correctly folded conformation. However, the correct folding of EC-Ret^{1–387} was further tested by

endoglycosidases assays. As recently reported, N-linked glycosylation of the extracellular domain of human Ret is not required for ligand binding, but it is relevant for the folding and the maturation of the protein [20,21]. We analysed the sensitivity of both EC-Ret^{wt} and EC-Ret¹⁻³⁸⁷ proteins to PNGase F and Endo H. It is well known in fact that PNGase F removes the oligosaccharide chains from all glycoproteins regardless of their folding state, whereas Endo H deglycosylates misfolded proteins that are retained in ER. EC-Ret¹⁻³⁸⁷ was incubated with either PNGase F or Endo H, and the reaction products were analysed by SDS-PAGE (Fig. 1). The same experiments were also carried out on native Ret for comparison. As shown in Fig. 1, the correct folding and maturation state of purified EC-Ret¹⁻³⁸⁷ were confirmed by its sensitivity to PNGase F and resistance to Endo H treatment. Comparable results were obtained with the EC-Ret^{wt} preparation (Fig. 1).

3.2. CLD4 and CRD are required for binding of EC-Ret^{wt} to GFR α 1/GDNF

By using the EC-Ret^{wt} and the deletion mutant EC-Ret¹⁻³⁸⁷ proteins, we determined whether the extracellular domain of Ret retains in vitro the ability to form a multiprotein complex with GDNF and GFR α 1 molecules. Equimolar amounts of recombinant GDNF, GFR α 1 and EC-Ret^{wt} (2 μ M final concentration each) were incubated in vitro in phosphate buffer and allowed to interact to obtain complex formation. In agreement with the methods reported in literature for the in vitro chemical cross-linking (see Ref. [14] as representative reference), low concentrations of the three protein components were chosen to avoid unspecific protein–protein interactions. Moreover, preliminary experiments were carried out to precisely determine the appropriate molar excess of reagents to limit the amount of cross-linking bonds and to avoid an excess of undesired surface labelling. As previously reported, in fact, a large number of chemical cross-links might perturb the native conformation of the complex, whereas modification of surface amino acids might greatly complicate the interpretation of the mass spectral data [15,16].

The cross-linking agent BS3, a bifunctional reagent linking amines to amines, thus selective for the N-terminal amino group and lysine side chains, with a spacer arm of 11.4 Å [22], was then added to the complex. The reaction was stopped by direct dilution with sample buffer, and the modified samples were fractionated by SDS-PAGE as shown in Fig. 2 (left panel, lanes 3–6). Isolated GFR α 1 and EC-Ret^{wt} proteins were also loaded onto the gel as specific electrophoresis mobility standards (lanes 2 and 7). Similar experiments were carried out using the truncated form of Ret, EC-Ret¹⁻³⁸⁷ (lanes 8–10).

Fig. 2 shows that the cross-linking reactions resulted in the production of covalently linked species that were named 1A, 1B, 1C and 1D according to their electrophoretic mobility (lanes 3–6). When the truncated EC-Ret¹⁻³⁸⁷ was used in the experiments, only three protein bands, namely, 2A, 2B and 2C, were observed (lanes 8 and 9). No evidence of a high molecular weight band corresponding to the 1D species was observed in these samples.

The protein bands were excised from the gel, reduced, alkylated and in situ digested with trypsin [23]. The resulting peptide mixtures were analysed by MALDI-MS following the mass fingerprinting procedure [15]. As an example, Fig. 3 shows the partial MALDI mass spectrum of the peptide mixture from band 1D, the specific complex only formed in the presence of native Ret protein. The mass spectral analysis revealed the occurrence of mass signals corresponding to linear peptides originated from the GDNF, GFR α 1 and Ret sequences, thus indicating that this band corresponds to a three-component covalent complex involving GDNF, GFR α 1 and EC-Ret^{wt}. Moreover, a series of signals were identified in the mass spectra that could not be assigned to any linear peptide. These signals were not detected in the mass mapping of isolated GDNF, GFR α 1 and Ret carried out as reference and were then interpreted as modified fragments. These modifications might have been generated either by surface labelling, i.e., BS3 modification of exposed residues or by intra- and/or intermolecular cross-links. The assignments of these mass signals were then performed on the basis of the mass values recorded in the spectra and the specificity of the trypsin digest [24], taking

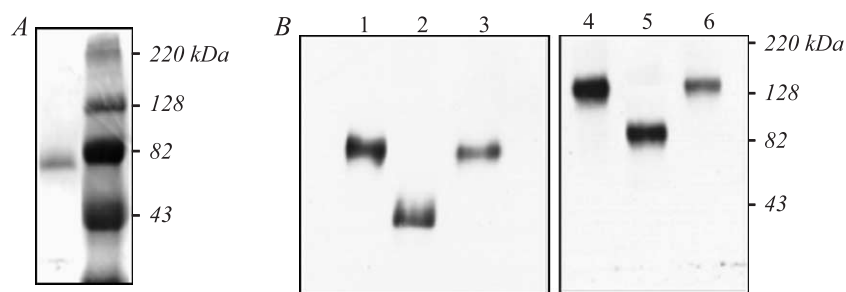


Fig. 1. Deglycosylation analysis of EC-Ret proteins. (A) SDS-PAGE (10% acrylamide) of a pure sample of EC-Ret¹⁻³⁸⁷ revealed by Coomassie Brilliant Blue staining; molecular-mass standards are shown. (B) EC-Ret¹⁻³⁸⁷ or EC-Ret^{wt} before (lane 1 or 4, respectively) and following treatment with PNGase F (lane 2 or 5, respectively) or Endo H (lane 3 or 6, respectively) immunoblotted with anti-Ret antibodies.

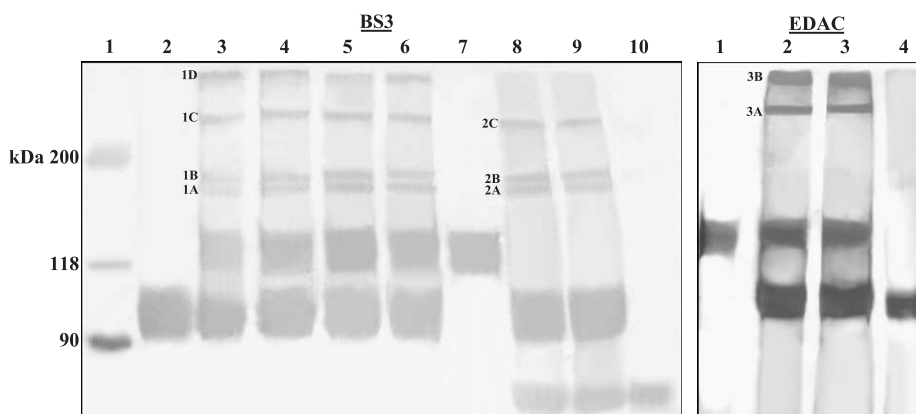


Fig. 2. Silver-stained SDS PAGE analysis of samples from BS3 and EDAC cross-linking experiments. The samples loaded on the gel are as follows: BS3 (left panel), markers (lane 1); GFR α 1 (lane 2); cross-linking reaction between EC-Ret^{wt}, GDNF and GFR α 1 (lanes 3–6, shown in triplicate); EC-Ret^{wt} (lane 7); cross-linking reaction between the truncated EC-Ret^{1–387}, GDNF and GFR α 1 (lanes 8–9, shown in duplicate); EC-Ret^{1–387} (lane 10). EDAC (right panel), EC-Ret^{wt} (lane 1); cross-linking reaction between EC-Ret^{wt}, GDNF and GFR α 1 (lanes 2–3, shown in duplicate); GFR α 1 (lane 4).

into account all the possible modifications occurring in the peptide. The mass searching of the modified peptides was carried out with a mass tolerance of 30 ppm, thus leading to univocal attributions of the signals, as summarised in Table 1. As an example, the signal at m/z 1045.6 was assigned to the GFR α 1 peptide 89–95 carrying a covalently linked BS3 moiety, while the signal at m/z 1320.6

revealed the occurrence of an intramolecular cross-link involving the fragments 89–91 and 92–97 of the GFR α 1 sequence. Following this procedure, a number of real intramolecular covalent bonds linking the three proteins were detected. The signal at m/z 1475.8 was assigned to the covalent cross-link between the peptide 91–97 of GFR α 1, oxidized at level of Met93, with the GDNF 1–4

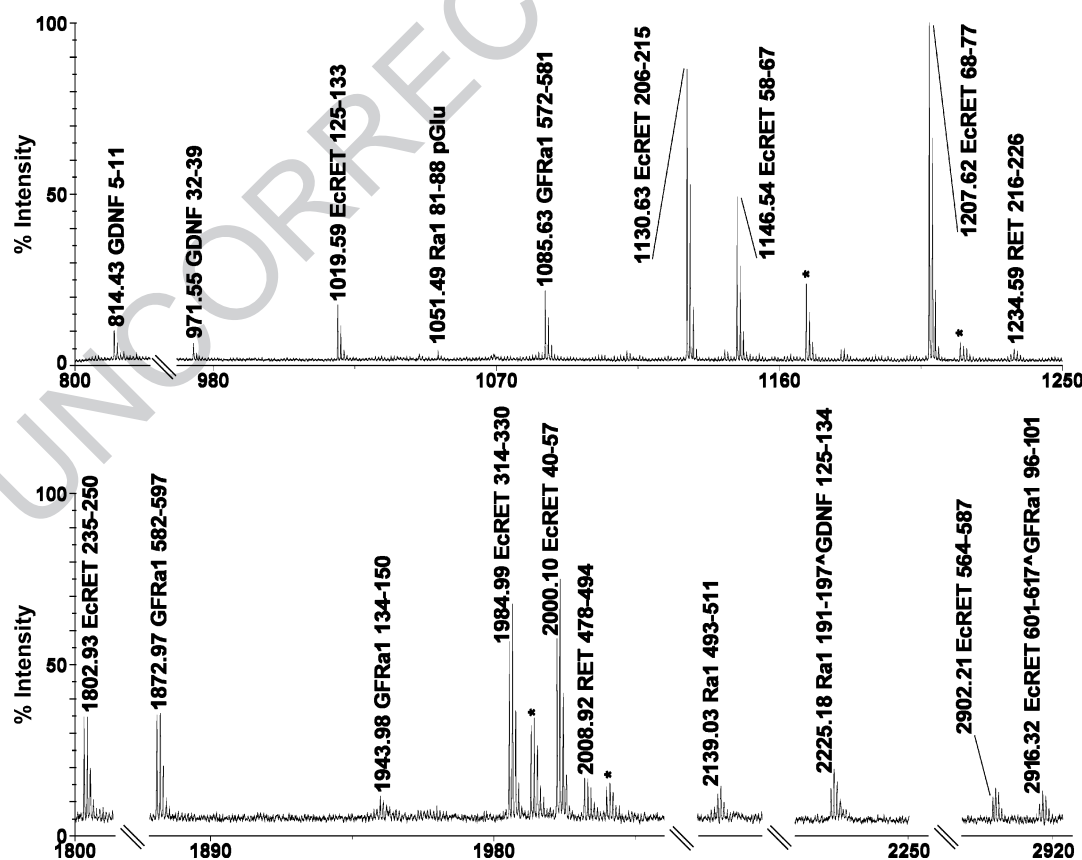


Fig. 3. Partial MALDI mass spectrum of the peptide digest from band 1D. Mass signals were assigned to the corresponding peptides within GDNF, EC-Ret^{wt} and GFR α 1 sequences on the basis of their mass values. Tripsin autoproteolysis peaks are marked with an asterisk.

Table 1

Mass values and corresponding fragments from the BS³ cross-linking experiment

Measured mass (<i>m/z</i>)	Modified peptides	Residues	Theoretical mass (<i>m/z</i>)	Found in band
1045.6	Rα1 89–95 ^{BS3} (internal)	Rα1 90 Rα1 94	1045.6	1D
1079.5	Rα1 89–91 ^{BS3} Rα1 92–95(ox)	Rα1 90 Rα1 94(95)	1079.5	1D
1320.6	Rα1 89–91 ^{BS3} Rα1 92–97(ox)	Rα1 90 Rα1 94(95)	1320.6	1A, 1B, 1C, 1D, 2A, 2B, 2C
1429.6	Rα1 92–97(ox) Rα1 191–194	Rα1 94(95) Rα1 191	1429.6	1A, 1B, 1C, 1D, 2B
1446.8	Rα1 91–97(ox) GDNF 28–31	Rα1 94(95) GDNF 29	1446.84	1D
1461.8	Rα1 89–95 ^{BS3} GDNF 28–31	Rα1 94(90,95) GDNF 29	1461.8	1D
1475.8	Rα1 91–97(ox) GDNF 1–4	Rα1 94(95) GDNF 1	1475.8	1D
1497.8	GDNF 1–4 GDNF 28–35	GDNF 1 GDNF 29	1497.8	1D
1699.8	Rα1 95–97 ^{BS3} Rα1 39–47	Rα1 95 Rα1 45	1699.8	1D
2225.2	Rα1 191–197 ^{BS3} GDNF 125–134	Rα1 191 GDNF 129	2225.1	1A, 1B, 1D, 2B, 2C
2916.3	Rα1 96–101 ^{BS3} RET 601–617	Rα1 97 RET 603	2916.3	1D

fragment. Moreover, the peak at *m/z* 2225.2 was identified as originated by the peptide 191–197 of GFRα1 covalently linked to the fragment 125–134 of GDNF, confirming that coreceptor/ligand interaction occurred through these regions of the two proteins. As a whole, a detailed examination of the data demonstrated that the GFRα1 region 91–97 is in close contact with the N-terminal portion of the GDNF molecule (region 1–31) via covalent cross-links involving either Lys94 or Lys95 of GFRα1 and the N-terminal amino group and Lys29 of GDNF. A further cross-link between the coreceptor and the ligand was established through Lys129 in the C-terminal portion of GDNF and either Lys191 or 194 of GFRα1, as reported in Table 1. Finally, a direct interaction between GFRα1 and EC-Ret^{wt} was established through a chemical linkage joining Lys97 of the coreceptor and Lys603 of Ret, as indicated by the signal at *m/z* 2916.3 involving the GFRα1 96–101 peptide and the EC-Ret^{wt} 601–617 fragment. It should be emphasised that no direct chemical cross-links between EC-Ret^{wt} and the GDNF ligand could be identified in the analysis.

The MALDI mass spectral analyses of the peptide mixtures from bands 1A, 1B and 1C occurring in the presence of the native EC-Ret^{wt} form showed the presence of signals corresponding to peptides generated from tryptic hydrolysis of the GDNF and GFRα1 molecules. No mass signals corresponding to Ret sequences were detected, indicating that these bands corresponded to heterodimeric complexes consisting of GDNF and GFRα1 proteins. The occurrence of three bands consisting of the same two proteins but showing different electrophoretic mobilities was interpreted as due to the different stoichiometry of the cross-linked complexes between GFRα1 and the GDNF

ligand. On the basis of their electrophoretic mobility referred to a calibration curve, the band 1C was tentatively attributed to a 2:2 GDNF/GFRα1 complex, whereas bands 1A and 1B were assigned to GDNF/GFRα1 complex with one GFRα1 molecule and different GDNF composition.

As reported in Table 1, several mass signals were detected in the spectra that corresponded to intermolecular covalently linked peptides, confirming that the region 191–197 of GFRα1 is directly joined to the C-terminal domain of GDNF. Interestingly, no linkages involving the N-terminal region of GDNF were detected.

Similar results were obtained in the analysis of the 2A, 2B and 2C bands that essentially confirmed the structural data obtained on the interaction between the ligand and the receptor occurring in the presence of the truncated EC-Ret^{1–387}.

The possible role of ionic interactions in the formation of the complex was investigated by selecting the acidic residues of the proteins for cross-linking formation. Following incubation of equimolar amounts of GDNF, GFRα1 and EC-Ret^{wt}, the protein carboxyl groups were activated by EDAC and allowed to react with amino groups to form zero-length intermolecular bonds [25]. The reaction mixture was then fractionated by SDS-PAGE (Fig. 2, right panel), and the protein bands were digested in situ. The tryptic mixture from 3A and 3B bands were analysed by MALDI-MS following the procedure as described, revealing the presence of linear peptides generated from enzymatic digestion of the GDNF, GFRα1 and Ret proteins, thus indicating the presence of a ternary complex. All the mass signals occurring in the spectra were attributed as described above. The analysis of the mass signals corresponding to cross-linked peptides essentially confirmed previous cross-linking

Table 2

Mass values and corresponding fragments from the EDAC cross-linking experiment

Measured mass (<i>m/z</i>)	Cross-linked peptides	Cross-linked residues	Theoretical mass (<i>m/z</i>)
1872.0	GFRα1 91–95(ox) GDNF 5–14	GFRα1 94(95) GDNF 13	1872.0
2726.2	GFRα1 92–97(ox) EC-Ret ^{wt} 478–494	GFRα1 94(95) EC-Ret ^{wt} 480	2726.3
3301.7	GFRα1 91–101(ox) GDNF 13–29	GFRα1 94(95) GDNF 24	3301.7

413 pattern, as illustrated in Table 2. The GDNF N-terminal
 414 portion is in close contact with the 91–97 region of the
 415 receptor GFR α 1, as demonstrated by the cross-links
 416 between Glu13 and Glu24 of GDNF with either Lys94 or
 417 95 from GFR α 1. Moreover, a covalent linkage joining the
 418 92–97 region of GFR α 1 and Glu480 of Ret was identified,
 419 thus further supporting the occurrence of a direct interaction
 420 between EC-Ret^{wt} and the coreceptor. The mass finger-
 421 printing analyses of the cross-linked bands corresponding to
 422 the GDNF/GFR α 1 complex essentially confirmed the
 423 results obtained with BS3.

424 3.3. EC-Ret^{I-387} does not compete with membrane-bound 425 Ret^{wt} for binding to GDNF

426 To further confirm that the CLD4 and CRD are required
 427 for binding to GFR α 1/GDNF complex, we investigated
 428 whether EC-Ret^{I-387} may act as a competitive inhibitor for
 429 membrane-bound Ret. Indeed, as previously reported [18],
 430 EC-Ret^{wt} inhibits the GDNF-induced Ret activity by com-
 431 peting the membrane-bound Ret receptor for ligand binding.
 432 We determined whether EC-Ret^{I-387} could interfere with the
 433 GDNF-induced stimulation of Ret. We took advantage of a
 434 PC12 derivative cell line expressing human Ret^{wt} (PC12/wt).
 435 As shown in Fig. 4A, in the presence of soluble GFR α 1,
 436 GDNF stimulates Ret tyrosine phosphorylation (compare
 437 lane 2 to lane 1). Treating cells with increasing amounts of
 438 EC-Ret^{I-387} did not affect the Ret phosphorylation (compare
 439 lanes 3 and 4 to lane 2), whereas EC-Ret^{wt}, used as a positive
 440 control, greatly decreased Ret tyrosine phosphorylation level,
 441 almost reaching the basal values (compare lanes 5 and 6 to
 442 lane 2). Accordingly, phosphorylation of the Ret downstream
 443 effector, ERK, was undetectable in the absence of the ligand
 444 (Fig. 4B, lane 1) and strongly stimulated by GDNF in the
 445 presence of soluble GFR α 1 (lane 2), thus suggesting the
 446 integrity of the signal transmission pathway. The truncated
 447 EC-Ret^{I-387} was not able to inhibit the GDNF-induced ERK
 448 phosphorylation (lanes 3 and 4), whereas, as expected, EC-
 449 Ret^{wt} behaved as a potent inhibitor (lanes 5 and 6).

450 As an appropriate control, we verified that the 10-min
 451 treatment of both EC-Ret^{wt} and EC-Ret^{I-387} at 37 °C in
 452 RPMI medium at a final concentration of 2 μ g/ml did not
 453 cause protein aggregation or misfolding of the proteins. The
 454 elution profile from a Superdex 200 column of the treated
 455 proteins (Fig 4C) gave rise to a symmetric chromatographic
 456 peak, with an apparent molecular mass compatible with the
 457 size of 120 and 80 kDa for EC-Ret^{wt} and EC-Ret^{I-387},
 458 respectively, thus indicating the correct conformation state
 459 of the molecules. The same results were obtained at a final
 460 concentration of 4 μ g of protein/ml of culture medium (not
 461 shown).

462 These findings indicate that the presence of CLD4 and
 463 CRD in the extracellular region are needed to compete Ret
 464 receptor for binding to GDNF.

465 We thus determined the biological effects of both EC-
 466 Ret^{wt} and EC-Ret^{I-387} proteins on Ret-dependent cell

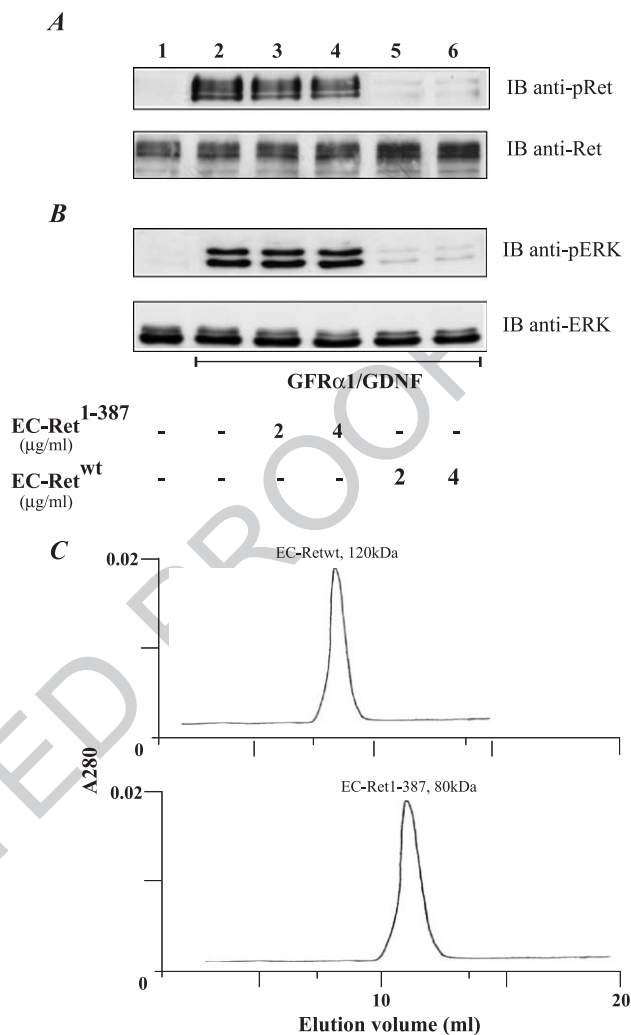


Fig. 4. Effect of EC-Ret proteins on Ret-mediated signaling. (A) PC12/wt cells (350,000 cells/6 cm plate) were starved for 4 h and then treated for 10 min with preincubated mixtures (5 ml final volume of RPMI medium), including GDNF (1.6 nM), soluble GFR α 1 (1.6 nM) and EC-Ret^{I-387} or EC-Ret^{wt} at the indicated concentrations. Cell lysates were immunoblotted with anti-phosphoRet antibodies (upper panel), the same filter was stripped and hybridized with anti-Ret antibodies (lower panel). (B) Cell lysates from cells treated as in panel (A) were immunoblotted with anti-phosphoERK antibodies (upper panel); the same filter was stripped and hybridized with anti-ERK antibodies (lower panel). (C) EC-Ret^{wt} and EC-Ret^{I-387} were treated for 10 min at 37 °C in RPMI medium (2 μ g/ml final concentrations) and then loaded onto a Superdex 200 High Load gel filtration column. The molecular sizes of EC-Ret^{wt} and EC-Ret^{I-387} are indicated.

467 differentiation. To this aim, we measured the GDNF-
 468 dependent neurite outgrowth in PC12- α 1/wt cells that stably
 469 express both human Ret and GFR α 1. As shown in Fig. 5A
 470 and B, cells extended long neuritic processes in response to
 471 1-day exposure to GDNF (b) in comparison with the
 472 nonstimulated control cells (a). Treatment of the cells with
 473 EC-Ret^{wt} (c) but not with EC-Ret^{I-387} truncated protein (d)
 474 significantly reduced the number and length of neurites. To
 475 further monitor the cell differentiation, we determined the
 476 extent of induction of VGF by GDNF stimulation.
 477 Consistently with the phenotypical effects reported above,

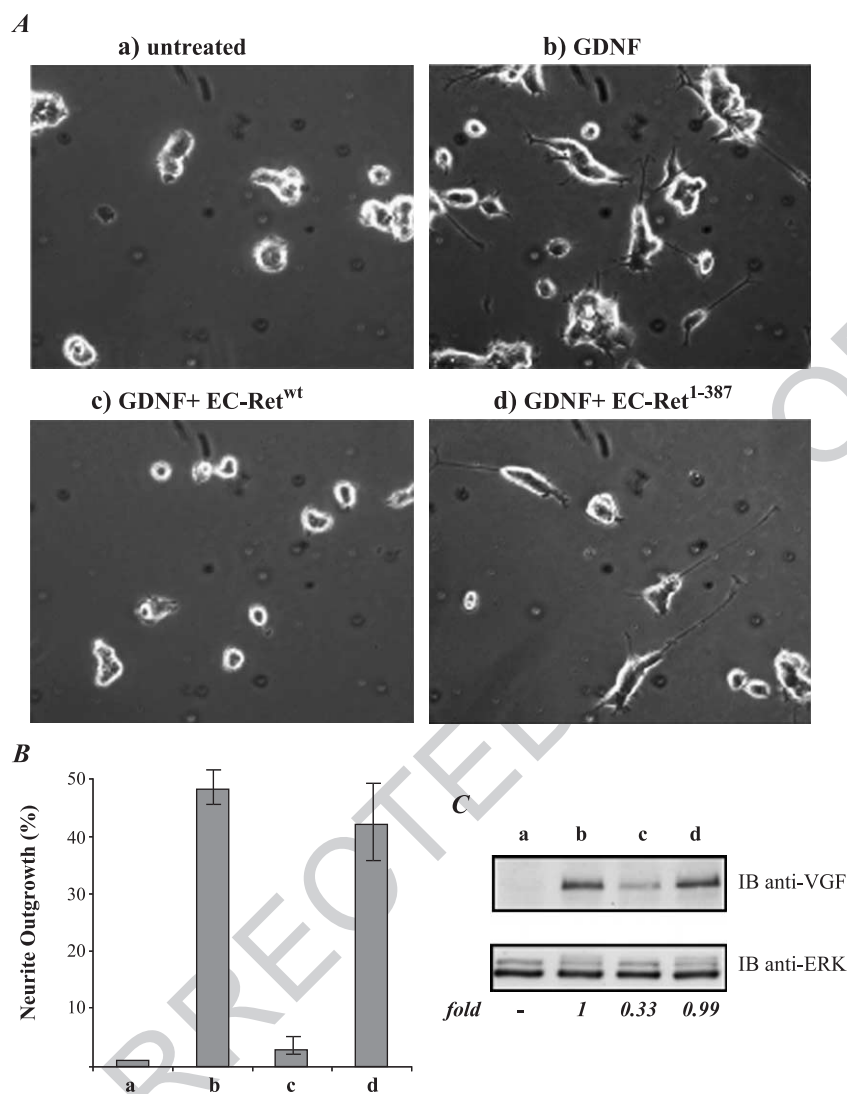


Fig. 5. Effect of EC-Ret proteins on GDNF-induced differentiation of PC12- α 1/wt cells. (A) Cells were left nonstimulated (a) or stimulated with GDNF alone (b) or together with EC-Ret^{wt} or EC-Ret¹⁻³⁸⁷ proteins (c and d, respectively). Following 24 h of GDNF treatment, the percentage of neurite outgrowth was calculated. (B) The data were expressed as percentage of neurite-bearing cells/total cells counted. Each experiment was repeated a minimum of three times. (C) Cell lysates were immunoblotted with anti-VGF and, to confirm equal loading, with anti-ERK antibodies as indicated. The fold values over control set to 1 (lane b) have been calculated using the NIH Image Program.

478 treatment with EC-Ret^{wt}, but not with the EC-Ret¹⁻³⁸⁷
 479 protein, strongly inhibits the GDNF-dependent stimulation
 480 of VGF expression (Fig. 5C).

481 4. Discussion

482 Given the obvious difficulty to solve the 3D structure of
 483 the Ret/GFR α 1/GDNF ternary complex, a need of structural
 484 and functional information is still required to identify the
 485 regions engaged in the interactions within the complex. The
 486 availability of soluble forms of Ret extracellular domains,
 487 GDNF and GFR α 1 molecules allowed us to directly study
 488 the interactions among these components in vitro, using
 489 highly homogeneous proteins. We performed cross-linking
 490 experiments involving the purified human Ret extracellular

portion together with the entire GDNF and GFR α 1
 molecules followed by MALDI mass spectrometry finger-
 printing analyses to obtain a direct identification of the
 chemical linkages joining Ret, the ligand and the coreceptor
 molecules. In these experiments, we used two recombinant
 Ret protein forms, the first corresponding to the entire Ret
 receptor extracellular portion (EC-Ret^{wt}) and the other one
 to the three first N-terminal domains (CLD1–3) of Ret (EC-
 Ret¹⁻³⁸⁷) lacking the CLD4 and CRD.

The results presented here show that, following cross-
 linking experiments in the presence of EC-Ret^{wt}, formation
 of a three-component complex occurred that involved Ret,
 GDNF and GFR α 1 molecules. The pattern of chemical
 cross-links observed in the analysis of this complex is
 reported in Fig. 6. On the contrary, formation of the three-
 component complex could not be detected when the

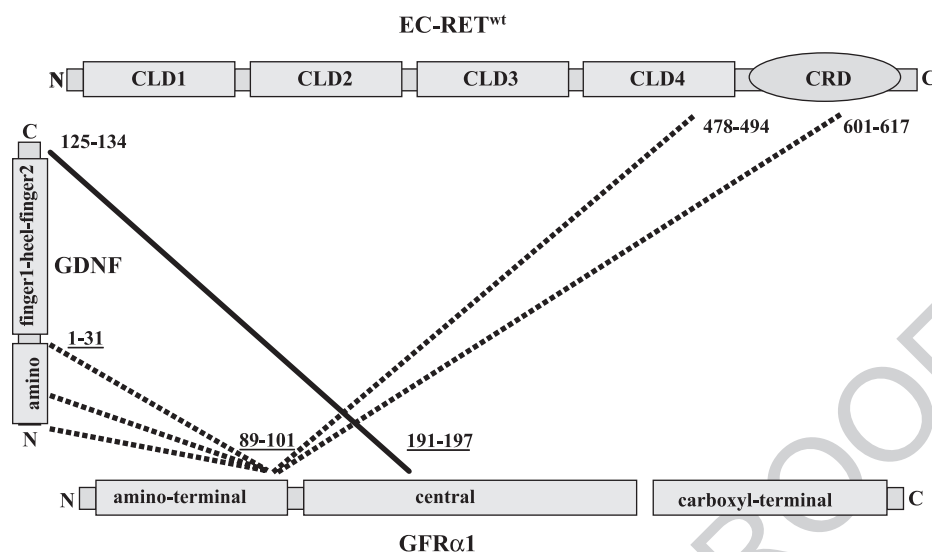


Fig. 6. Schematic representation of covalent cross-links joining GDNF, EC-Ret^{wt} and GFRα1. Lines represent cross-linking bonds; dotted lines indicate that cross-links occur only if EC-Ret^{wt} was present in the complex. Regions concerned by cross-links are indicated by the respective amino acids and are underlined. Domains of GDNF and GFRα1 proteins are labeled accordingly to Refs. [9,10], respectively.

recombinant truncated form of Ret (EC-Ret¹⁻³⁸⁷) was used in the experiments. Moreover, formation of two-component complexes involving GDNF and GFRα1 interacting with different stoichiometry was observed in the presence of both native and truncated Ret forms. The panel of covalent linkages found between the ligand and the coreceptor was only slightly different as compared to that observed in the three-component complex. Finally, no complexes between Ret and GDNF or between Ret and GFRα1 could be detected in our analysis.

On the other hand, the current model for GDNF signaling proposes that direct binding of GFRα1 to GDNF is a required event to stimulate Ret and that Ret binding to GFRα1 increases the affinity of GFRα1 for GDNF [8]. Although the absence of complexes between Ret and GDNF could not rule out any substantial physical contacts between these proteins, the data are in good agreement with the need of GFRα1 in the same complex to stimulate Ret activity [8]. Applying a similar logic, it is reasonable to assume either that Ret may bind uniquely to GFRα1 following conformational changes taking place in the coreceptor upon binding GDNF or, alternatively, that a two-component complex of Ret with GFRα1 can take place but at a lower stability with respect to the three-component complex involving Ret, GDNF and GFRα1. In the latter case, it would mean that the affinity of Ret for the preformed GFRα1/GDNF complex is higher than that of Ret for GFRα1 or GDNF kept separately.

As recently reported, the central domain of GFRα1 is necessary to dictate the ligand binding specificity and is critical for GDNF-induced Ret activation [10–12]. In agreement with the reported importance of this central region of GFRα1 for ligand binding, we found that the region 191–197 of GFRα1 is directly joined to GDNF. Interestingly, this GFRα1 region interacts with the 125–134 region in the C-terminal domain of GDNF, which is

neighbouring to a surface that includes four negatively charged and four hydrophobic residues. The same region has been reported to form a hot spot for GDNF binding to the GFRα1 coreceptor [8]. Although GFRα1 binds the C-terminal domain of GDNF both in the presence and in the absence of Ret in the complex, an additional contact between the 91–97 region of the GFRα1 molecule and the N-terminal (1–31 residues) of the GDNF molecule occurs exclusively if Ret is present in the same complex (see Fig. 6). This finding suggests that binding of Ret to GFRα1/GDNF complex induces a conformational change in the complex, causing the first residues of the N-terminal region in the GDNF molecule to contact a newly exposed GFRα1 region encompassing residues 91–97. This model is well supported by the assumption that the first 36 residues in the N-terminus of GDNF represent a highly flexible region since they could not be resolved in the crystal structure [26]. This large N-terminal region is well conserved between rat and human, however, despite the high structural homology with neurturin, artemin and persephin, it is unique to GDNF. On the other hand, by transient expression assays, it has been shown to be dispensable for stimulation of Ret downstream signaling [9], thus raising the question of understanding the function for this region in the complex. A plausible interpretation for the results presented suggests that this region could be implicated in the formation of a high-affinity GDNF-binding site on GFRα1 that is observed only in the presence of Ret [7].

The observation that a GFRα1 region (91–97), including residues located in the third predicted α-helix of the N-terminal domain, interacts with GDNF is in apparent discrepancy with the dispensability of this region for GDNF-induced stimulation of Ret. Indeed, variants of the coreceptor molecule truncated of the entire N-terminal domain still retain the ability to bind GDNF although at

low efficiency, thus indicating that this region of GFR α 1 is not needed for ligand binding [11]. On the other hand, cross-linking experiments with either of the specific reagent used (BS3 and EDAC) strongly support the relevance of this interaction. However, it should be pointed out that the interaction of the N-terminal portion of GFR α 1 with the N-terminal region of GDNF only occurs following formation of the ternary complex in the presence of Ret. On the contrary, the contact between the GFR α 1 191–197 region with the GDNF C-terminal portion was observed regardless of the presence of Ret. Therefore, it is reasonable to assume that the N-terminal portion of GFR α 1 is required to stabilize the GFR α 1/GDNF complex, thus explaining the suboptimal ligand binding efficiency reported for the deletion mutants [11].

When the Ret, GDNF and GFR α 1 three-component complex was examined following cross-linking experiments, we found that Ret is covalently linked to GFR α 1, but not to GDNF. In particular, the N-terminal portion of GFR α 1 joined to the CLD4 and CRD domains, located proximal to the transmembrane domain, was involved in these interactions. On the other hand, no interaction involving the three first CLDs domains of Ret (CLD1–3) was found with neither cross-linking reagents. Consistently, we show that EC-Ret^{1–387} protein, corresponding to the CLD1–3 domains and thus lacking both the CLD4 and CRD, does not bind to GFR α 1/GDNF complex since no covalent cross-link involving EC-Ret^{1–387} was found. The structural evidence that CLD4 and CRD are required for binding of Ret to GFR α 1/GDNF complex was strongly confirmed by functional data on living cells. Indeed, in PC12- α 1/wt cells, the stimulation by GDNF of both Ret intracellular signaling and cell differentiation were strongly inhibited by EC-Ret^{wt}, but not by EC-Ret^{1–387} protein, thus indicating that the truncated EC-Ret^{1–387} that lacks both CLD4 and CRD is not able to act as inhibitory competitor for the membrane-bound Ret receptor.

As a whole, these results demonstrate that CLD4 and CRD are dispensable for the correct folding and maturation of Ret extracellular domain, but they are necessary for both structural and functional binding of Ret to the GFR α 1/GDNF complex.

By using the homologue-scanning mutagenesis analysis between the extracellular domains of human and *Xenopus* Ret, it has been reported [13] that the existence of a surface spanning the three N-terminal CLDs (CLD1–3) of human Ret that is required for ligand binding. This finding led to a model that must presuppose a high flexibility of the Ret extracellular portion, allowing Ret to reach the binding sites on GFR α 1 anchored to the plasma membrane. A strong implication of our results resides in the possibility to reformulate this interaction model, suggesting that requirement of CLD1–3 elements for binding is rather indirect, while a physical interaction occurs between the C-terminal portion of Ret (CRD and CLD4 domains) and the GFR α 1/GDNF complex in close contact with the cell membrane.

The first three N-terminal domains of Ret either would contribute to stabilize the complex once formed or might be necessary for Ret to adopt a conformation competent for the assembly of a functional complex.

In conclusion, we show that two small regions of GDNF are engaged in close physical interactions with GFR α 1, one of which requires the presence of Ret in the same complex to be positioned close to the GFR α 1. Conversely, the complex between Ret and GFR α 1/GDNF is stabilized by major contacts involving the N-terminal domain of GFR α 1 and the CLD4 and the CRD domains of Ret. These domains are necessary for both structural and functional binding of the receptor to the complex. Results make it possible to suggest a model for the molecular basis of ligand-mediated Ret stimulation in attempts to design minimal agonists of the GDNF molecule to be used both as therapeutics and diagnostic agents for neurodegenerative diseases.

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